UNIVERSITY OF CALIFORNIA, MERCED

Microbial ecology and methane dynamics of high-elevation lakes

Dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Environmental Systems

by

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Dedication

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Curriculum Vitae

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Perez-Coronel, E; Beman, J.M.
Biogeochemical and multi-omic evidence for multiple mechanisms of paradoxical methane production in freshwater lakes. In review.
https://www.biorxiv.org/content/10.1101/2020.07.28.225276v1

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Experimental warming can increase aerobic methane production and oxidation in surface waters of freshwater lakes. Manuscript in preparation.

Beman, J.M; Vargas, S; Wilson, J; Perez-Coronel, E; Yu, A; Vazquez, S; Cairo, A; Karolewski, J; Wankel, S.
Constraining oxygen consumption by nitrite oxidation in oceanic oxygen minimum zones. In review. https://www.biorxiv.org/content/10.1101/2020.05.26.115402v1

Beman, J.M; Vargas, S; Vazquez, S; Wilson, J; Yu, A; Cairo, A; Perez-Coronel, E.
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Abstract of the Dissertation

Microbial ecology and methane dynamics of high-elevation lakes

By

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University of California, Merced 2020
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Methane (CH_4) is the second most important greenhouse gas after CO_2 contributing to climate change. Some ecosystems, such as high-elevation lakes, have been overlooked in their contribution to the CH_4 budget and require additional study as they may be disproportionately affected by climate change via increases in average temperatures and changes in seasonal variability. Through extensive sampling and experimentation, this research examined how environmental variation influenced microbial CH_4 production and oxidation in the surface waters of high-elevation lakes in the Sierra Nevada, and identified potential sources of aerobic CH_4 production. Main findings include that seasonality in CH_4 concentrations and fluxes is substantial in freshwater lakes such that predicted increases in temperature, and changes in nutrient loading and dissolved organic carbon may affect overall CH_4 emissions from high-elevation lakes. Aerobic CH_4 production was carried out in its majority by members of the *Comamonadaceae* family through the lysis of the C-P bond of methyl phosphonate and through a new potential mechanism by Proteobacteria and Cyanobacteria during (bacterio)chlorophyll metabolism. Moreover, these potential aerobic CH_4 mechanisms can be sensitive to warming as shown by the upregulation of functional genes involved in these processes, but the magnitude of the response varied among experiments which suggests that warming alone cannot explain disparities in aerobic CH_4 cycling. Overall, these results indicate that CH_4 cycling in high-elevation lakes is highly dynamic and heavily influenced by environmental variations, which highlights the importance of further studying these lake ecosystems as our planet changes.

1 Introduction

Methane (CH_4) is an important greenhouse gas that has gained attention of biogeochemists over the past century due to its impact in global climate change. Methane contributes 20% of the warming produced by all well-mixed greenhouse gases (Saunois et al., 2016) and has 28 times the global warming potential of CO_2 (IPCC 2014). The atmospheric concentration of CH_4 has increased over 150% since pre-industrial times from 715 ppb to the present concentration of 1873 ppb (Bousquet et al., 2006; Saunois et al. 2016; NOAA ESRL *www.esrl.noaa.gov/gmd/ccgg/trends_ch4/*). The current global budget of atmospheric CH_4 of about 500–600 Tg CH_4 $year^{-1}$ is determined by the balance of methane sources and sinks which varies slightly every year but has a general increasing trend (Wang et al., 2004; Saunois et al. 2016). These sources and sinks can be biotic and abiotic and are constantly impacted by anthropogenic activities and by natural phenomena. Since CH_4 holds such an important role in the radiative forcing of the planet, the study of the CH_4 component in the carbon cycle is critical to understand further alterations in global climate.

It has been estimated that about 60% of CH_4 emissions are caused by anthropogenic activities whereas 30% comes from natural sources (Nisbet et al., 2014; Saunois et al. 2016). Main anthropogenic sources of CH_4 are coal industries, agriculture, landfills, natural gas wells and pipelines and biomass burning (Nisbet et al., 2014). Natural CH_4 emissions include those from terrestrial and aquatic sources such as tropical and boreal forests and marine and freshwater ecosystems, while wetlands are recognized as the world's largest natural methane source (Bousquet et al., 2011; Saunois et al. 2016). Moreover, freshwater lakes have a disproportional contribution of CH_4 emissions compared to their volume (Bastviken et al., 2008; Bastviken et al., 2011; DelSontro et al., 2018) and an active effort to understand the CH_4 producing and consuming microbial processes in these ecosystems is ongoing.

Freshwater lakes represent an important source of atmospheric CH_4, their contribution is estimated to be about 6-16% of the natural CH_4 emissions while only accounting for 0.9% of the Earth's surface (Bastviken et al., 2004). Additionally, man-made reservoirs contribute to 18% of the CH_4 anthropogenic emissions (Louis et al., 2000). In freshwater lakes, most of the CH_4 production is carried out by microorganisms identified as methanogens and occur in anoxic sediments from where CH_4 can later be transported through ebullition (direct flux from sediments to the atmosphere) or diffusion (Bastviken et al., 2004). Through diffusion, CH_4 enters the water column and it is mostly oxidized by methanotrophic microorganisms (30-99%) while the remaining will reach the upper water column and can be released to the atmosphere by diffusive emission (Bastviken et al., 2004). However, during periods of low turn-over in stratified lakes, CH_4 can be accumulated in the anoxic layer of the water column and released during lake mixing (Riera et al., 1999). Additionally, CH_4 can also by emitted through plants in lakes with emergent vegetation (Segers, 1998; Bastviken et al., 2002).

Conventionally, CH_4 production was known to occur only via methanogenesis in anoxic sediments or during conditions of lake stratification that can generate anoxic zones in the water column (West et al., 2016). However, it was recently discovered that CH_4 production in oxygenated water columns is a widespread process in lakes even though methanogenesis is assumed to be inhibited by oxygen (Hoehler et al., 2018). The underlying mechanisms of how CH_4 can be produced and accumulated in aquatic water columns are currently under study but it appears to be a significant contributor to the total lake CH_4 efflux (Bogard et al., 2014). The "methane paradox" as this phenomenon is often referred as, has important implications for lake CH_4 cycling because CH_4 produced in the water column can more easily reach the surface (Grossart et al., 2011) and it is less likely to be oxidized due to light inhibition of methane oxidation (Murase & Sugimoto, 2005; Thottathil et al., 2018). Several mechanisms for paradoxical CH_4 production have been proposed such as production in anoxic microsites—fecal pellets, detritus, and the gastrointestinal tracts of larger organisms such as fish or zooplankton (Oremland 1979; Traganza et al. 1979; Angelis & Lee 1994; Karl & Tilbrook 1994) or on the phycosphere (Grossart et al., 2011). Other proposed hypotheses include methanogens with oxygen-tolerant or detoxifying pathways that could aid in CH_4 production in the presence of oxygen (Angle et al., 2017). There are some mechanisms of aerobic CH_4 production in which methanogens take no part, such as the microbial utilization of methyl phosphonate and the consequent breakdown of the C-P bond in this molecule which results in CH_4 release (Karl et al., 2008). This is the prevailing mechanism of CH_4 production in the ocean (Karl et al., 2008) and it has been observed in freshwater lakes as well (Yao et al., 2016; Wang et al., 2017). Most recently, Bižić et al., (2020) work indicated that Cyanobacteria can directly produce methane during photosynthesis through an unknown pathway. Overall, it appears like CH_4 production is more widespread and diverse that previously known.

The balance between microbial CH_4 production and consumption determines the overall CH_4 emissions from lakes (Bastviken et al., 2004), regardless of the CH_4 production pathway, and, as any other microbial mediated process they can be highly sensitive to changes in temperature (Marotta et al., 2014). However, it is unclear if the multiple potential pathways of CH_4 production and CH_4 oxidation will respond to warming in a similar way. In general terms, methanogenesis is expected to increase with higher temperature, while CH_4 oxidation does not increase with temperature as consistently as methanogenesis. CH_4 oxidation is instead more dependent on other environmental conditions, such as CH_4 and oxygen concentrations (Lofton et al., 2013; Thottathil et al., 2018). The response to warming of paradoxical CH_4 mechanisms is completely unknown, however, it is critical to further characterize aerobic CH_4 production and how it responds to environmental change to accurately predict future CH_4 emissions from freshwater systems. Nonetheless, current evidence on the differential response to warming of CH_4 production and consumption and ecosystem-wide CH_4 emissions suggests that warmer temperatures will exacerbate overall lake CH_4 emissions (Yvon-Durocher et al., 2014; Sepulveda-Jauregui et al., 2018; Thottathil et al., 2019), generating a positive climate feedback from freshwater lakes over the following decades.

While temperature is expected to increase throughout the globe, some ecosystems are expected to be more sensitive to warming (IPCC, 2014). Such is the case of high-elevation regions, which are predicted to experience faster changes in temperature derived from increased air temperatures, reduced ice cover in lakes and snow-albedo and changes in cloud cover (Mountain Research Initiative EDW Working Group et al., 2015; O'Reilly et al., 2015; Sadro et al., 2019). These impacts will result in changes in seasonal patterns such as longer growing seasons due to reduced snowpack and ice cover on lakes (Moser et al., 2019) which will undoubtedly affect CH_4 lake emissions. While rarely measured, high-elevation lakes can exhibit significant CH_4 concentrations and fluxes (McCrackin and Elser 2011; Pighini et al., 2018) but their contribution to the CH_4 budget may be underrepresented in comparison to tropical or boreal lakes due to a lack of measurements (Saunois et al., 2016). There are thousands of such lakes in the Sierra Nevada of California (Melack and Stoddard 1991; Sickman et al. 2003) and, this region already displays a long-term warming trend (Sadro et al., 2019) which is expected to result in increased snow-albedo feedbacks and changes in type of precipitation (Walton et al. 2016; Sun et al. 2019). Changes in the precipitation trends combined to increased atmospheric deposition will alter nutrient inputs into high-elevation lakes which could have significant impact in trophic dynamics of these ecosystems (Williams et al., 2001; Sickman et al., 2003; Elser et al., 2009; Aciego et al., 2017). An improved understanding of the effects of environmental variation on CH_4 microbial cycling in high-elevation lakes will help us account for future changes in CH_4 fluxes and better predict long-term climate trends.

1.1 Dissertation organization and overview

This dissertation is organized in five chapters. Chapter 1 includes background information that informed this research project. The results of this research were written as three different manuscripts and are shown here as Chapter 2, 3 and 4. Chapter 2 describes how environmental changes such as changes in temperature, nutrient status and dissolved organic carbon concentrations impact CH_4 cycling of five high-elevation lakes over an elevation gradient over the course of two years. Chapter 3 explores the mechanisms of aerobic CH_4 production that were active in these lakes through a series of experiments and different treatments such as the addition of a methanogenesis inhibitor and increase or absence of light intensity. This chapter shows that methane is being produced under oxic conditions through established as well as novel paradoxical CH_4 production mechanisms. Chapter 4 investigates the response to warming of non-methanogenic CH_4 production and CH_4 oxidation through extensive experimentation and found that warming overall had a positive effect on abundance of functional genes related to CH_4 cycling processes. Finally, Chapter 5 summarizes the main findings of this dissertation and provides some insights and considerations for future research on CH_4 cycling of freshwater lakes.

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2 Seasonal methane dynamics in high-elevation lakes in the Sierra Nevada California: the role of elevation, temperature, organic matter, and inorganic nutrients

2. 1 Abstract

Freshwater lakes are important but poorly constrained sources of methane (CH_4) to the atmosphere due to high, but variable, rates of CH_4 production, as well as limited and inconsistent measurements worldwide. High-elevation lakes have been particularly overlooked—despite their large numbers in mountain ranges around the world, and despite CH_4 dynamics at high elevations may be altered by rapid increases in temperature due to climate change. We examined variations in surface CH_4 concentrations and diffusive fluxes, temperature, dissolved organic matter (DOC), and inorganic nutrients in five montane lakes spanning multiple elevations in the Sierra Nevada of California. Over two years, we found strong and consistent seasonality in CH_4 concentrations in lakes; higher concentrations were typically observed in the warmest months and lower concentrations in fall. Changes in CH_4 concentrations were significantly related to temperature in the majority of the individual lakes (r^2 = 0.43-0.81) and related to elevation (r^2 = 0.39) and DOC (r^2 = 0.30) across lakes. Methane concentrations in lakes at elevations <3000 m were strongly related to temperature, nitrite concentrations and elevation (r^2 = 0.90), whereas at elevations >3000 m, CH_4 correlated with dissolved inorganic nitrogen to dissolved inorganic phosphorus ratios and elevation (r^2 = 0.48). Our results expand on our understanding of temporal variations in CH_4 and demonstrate substantial seasonality in CH_4 concentrations and diffusive fluxes in freshwater lakes—suggesting that temporal variation should be considered in large-scale estimates, and may be a predictable function of elevation, temperature, organic matter, and nutrients.

2.2 Introduction

Methane (CH_4) is a potent greenhouse gas with great influence on the world's climate (Hoegh-Guldberg et al. 2018; Reay et al. 2018). While anthropogenic emissions have increased atmospheric CH_4 concentrations over the last century, an important baseline contribution of CH_4 to the atmosphere comes from natural ecosystems (Conrad 2009; Kirschke et al. 2013). Methane emissions from freshwater lakes are a particularly significant, but poorly constrained, component of natural CH_4 emissions. For example, the contribution of freshwater ecosystems (lakes and rivers) to the global CH_4 budget is estimated to range from 12% to 32% (with uncertainties ranging from 2% to 47%) of natural emissions (Saunois et al., 2016). More specifically, current estimates of the global CH_4 total emissions from freshwater lakes and impoundments range an order of magnitude, from 69 to 204 Tg CH_4-C yr^{-1} (DelSontro et al., 2018). These uncertainties stem partly from the substantial spatial and temporal variability in CH_4 cycling across and within freshwater lakes, as well as significant under-sampling of this variability. For instance, only a few hundred lakes have been sampled for CH_4

emissions out an estimated total of 304 million lakes globally (Downing 2009; Bastviken et al. 2011), and only a handful of studies have measured CH_4 variation over time (Xing et al. 2005; Casper et al. 2009; Palma-Silva et al. 2013; Natchimuthu et al. 2014; Martinez-Cruz et al., 2015). Clearly, additional studies are needed of the spatial and temporal heterogeneity in CH_4 emissions from these ecosystems, as well as their potential controls (Tranvik et al. 2009; Bastviken et al., 2011; Reay et al. 2018).

Variations in CH_4 concentrations in lake water columns are driven by microbial CH_4 production (predominantly via methanogenesis in sediments) and consumption via microbial CH_4 oxidation in surface sediments and the water column (Bastviken et al., 2004). Both of these processes can be altered by changes in environmental conditions and, in general, temperature seems to have a strong positive effect on both of them (Zeikus & Winfrey 1976; Duc et al. 2010; Palma-Silva et al. 2013; Lofton et al. 2014; Marotta et al. 2014; Sepulveda-Jauregui et al. 2018). Moreover, ecosystem-level analyses seem to indicate that as temperatures rise, CH_4 lake concentrations and fluxes will also increase (Natchimuthu et al. 2014; Yvon-Durocher et al. 2014; Rasilo et al. 2015). However, much of our understanding of temperature regulation of CH_4 concentrations in freshwater is derived from laboratory- or field-based experimental temperature manipulations. While these studies are extremely useful for isolating the effects of temperature from other variables, *in situ* temperature may vary in concert with other seasonally changing environmental properties, such as lake organic carbon (C) inputs and nutrients concentrations. For example, the quantities and types of organic C present in lakes during the year influence CH_4 production (R. Conrad, 1999), and this in turn is regulated by nutrient availability (Sepulveda-Jauregui et al., 2018). For CH_4 consumption, on the other hand, temperature may only play a role when neither CH_4 nor DO concentrations are limiting (Harrits & Hanson 1980; Liikanen et al. 2002; Martinez-Cruz et al. 2015). Collectively, these environmental factors may interact to influence CH_4 production, consumption, and emissions. The degree to which temperature—versus other environmental factors—affects CH_4 emissions from lakes remains largely unknown. Further characterization of this response is necessary as it could result in a positive climate feedback as global temperatures increase.

High-elevation regions are predicted to experience increased air temperatures, reduced lake ice cover, reduced snow-albedo, and changes in cloud cover as a result of climate change (Mountain Research Initiative EDW Working Group et al. 2015; O'Reilly et al. 2015; Sadro et al. 2019). Temperate montane lakes therefore could be disproportionately affected by climate change—including both increases in average temperatures, as well as changes in seasonal variability (e.g., reduced ice cover and a longer growing season). Importantly, high-elevation lakes can exhibit high CH_4 concentrations and fluxes (McCrackin & Elser, 2011), but CH_4 cycling is rarely characterized in these lakes. Their contribution to the CH_4 budget may be underrepresented in comparison to tropical or boreal lakes due to a lack of measurements (Saunois et al., 2016), even though they represent around 10% of lakes globally (Verpoorter et al., 2014). The Sierra Nevada of California is home to

thousands of such lakes (Melack & Stoddard 1991; Sickman et al. 2003), and this region already displays a long-term warming trend (Sadro et al., 2019). Snow-albedo feedbacks and changes in the type of precipitation will likely further increase warming and reduce snow pack melt in the decades to come (Walton et al. 2016; Sun et al. 2019).

Further reduction in snowpack melt and rain could also affect high-elevation lakes by altering nutrient inputs into freshwater lakes from snowmelt (Williams et al. 2001; Sickman et al. 2003). At the same time, increased nutrient loading from atmospheric deposition has been shown to alter algal communities and trophic dynamics in high-elevation ecosystems (Baron et al. 2000; Elser et al. 2007; Elser et al. 2009). Atmospheric deposition is a significant source of nutrients to the Sierra Nevada (Aciego et al., 2017) , where changes in nutrient limitation may already be occurring due to human activity in the adjacent San Joaquin Valley (Sickman et al., 2003). Such changes could consequently impact CH_4 cycling in lakes by altering organic C sources and quantities to these ecosystems (Tranvik et al. 2009; West et al. 2016; Reay et al. 2018; Moser et al. 2019). Finally, recent research indicates that the combination of increased temperature and nutrient concentrations can strongly enhance lake CH_4 production and ebullition (Davidson et al. 2018; Sepulveda-Jauregui et al. 2018). An improved understanding of the effects of environmental variation on CH_4 emissions in this ecosystem will help us account for future changes in CH_4 fluxes and better predict long-term climate trends.

We used large natural variations in temperature over time and with increasing elevation to determine the potential importance of temperature controls on CH_4 emissions from montane lakes. High-elevation lakes in the Sierra Nevada represent an ideal experimental system for examining temperature effects on CH_4 emissions within natural freshwater ecosystems because of these strong natural variations. Over two years, we quantified CH_4 concentrations and diffusive emissions in five lakes spanning an elevation gradient in the Sierra Nevada, California (Fig. 2.1). In addition to temperature variation, we measured two main categories of environmental variation that are likely to affect CH_4 emissions: nutrient (ammonium, nitrite, nitrate, phosphate) concentrations and availability and organic C production and properties (dissolved organic C and specific UV absorbance at 254 nm). Our aim was to answer the following research questions: 1) To what degree do CH_4 concentrations vary spatially over an elevation gradient and temporally over the seasons in high-elevation lakes, and how does this compare with other lake types? and 2) how much of the variation in CH_4 concentrations within and among lakes can be explained by seasonal fluctuations in environmental parameters (independently or collectively) such as temperature, nutrients and organic C concentrations, as well as lake intrinsic characteristics (e.g., elevation)? We hypothesized that temperature and nutrient concentrations would have the greatest effect on CH_4 concentrations in surface lake water, especially at higher elevations where temperatures are rapidly increasing, and landscapes characteristics contribute less allochthonous nutrient sources to lakes.

2.3 Materials and methods

2.3.1 Study site and sample collection

Five high-elevation lakes in the Sierra Nevada of California were selected based on preliminary data showing high microbial production and consumption rates and methanogen and CH_4-oxidizer abundances (Hayden & Beman, 2016). Lukens, Lower Cathedral, Upper Cathedral, Lower Gaylor, and Upper Gaylor Lakes have an elevation range of 2489 to 3185 m, and mean summer surface water temperature range of 12.8 to 17.2 ˚C (Fig. 2.1). Water samples were collected every ~2-3 weeks in the littoral and limnetic zones from July to November of 2016 and 2017 (sampling season) due to field site inaccessibility from December to June. Samples were collected at 0.1 m depth with a previously acid washed plastic or glass containers to measure: CH_4, nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) concentrations; and dissolved organic carbon (DOC). Temperature (T) and dissolved oxygen (DO) were measured on site using a ProODO YSI probe (YSI Inc., Yellow Springs, OH, USA).

2.3.2 Methane measurements

Methane concentrations were measured via headspace equilibration and gas chromatography. In brief, triplicate water samples were collected directly into 170 ml glass vials, capped with halogenated butyl stoppers, and crimped with aluminum seals to avoid gas loss. Twenty-five ml of water was then replaced with 25 ml of air collected on site, while air samples were collected into 12-mL Labco Exetainer vials (Labco Ltd., Lampeter, Ceredigion, UK). One hundred seventy mL glass vials containing water and headspace were shaken for 2 minutes to reach equilibration, and the headspace was collected with a gas-tight syringe; headspace gas samples were immediately transferred into 12-mL Exetainer vials. Triplicate samples were later analyzed using a Shimadzu GC-2014 gas chromatograph with flame ionization detection (FID) for CH_4 (Weiss, 1981). Samples were analyzed within 2-3 months of collection (a time period of up to 15 weeks has been tested and found to result in no significant change in CH_4 concentrations, with 2% or less decrease in 5 ppm CH_4 concentration from storage). Methane standards (Air Liquide, Houston, Texas USA) ranged from 1.5 to 333 ppm and bracketed every 15 samples; standard curve r^2 values ranged from 0.997 - 0.999 across different runs. Headspace CH_4 concentration measurements were then used to calculate CH_4 concentration in lake water based on Henry's law of equilibrium (Yamamoto et al. 1976).

2.3.3 Diffusive methane fluxes

Diffusive CH_4 fluxes were calculated with the following equation:

$$F = k\,(C_w - C_a),$$

where F is the flux, k is the gas transfer coefficient, C_w is the dissolved CH_4 concentration in the water, and C_a is the concentration of CH_4 in the air. The parameter k was estimated from wind-speed data collected from meteorological stations (White Wolf, Tuolumne Meadows, and Tioga Pass stations, each selected for their close proximity to Lukens, Cathedral, and Gaylor Lakes respectively) in Yosemite National Park maintained by the California Department of Water Resources (http://cdec.water.ca.gov) and the relationship developed by Cole and Caraco (1998) for low-wind speeds:

$$k_{600} = 2.07 + 0.215U_{10}^{1.7},$$

where k_{600} is the gas coefficient normalized to Schmidt number (Sc) 600, and U_{10} is the wind speed adjusted to 10 m following Amorocho and DeVries (1980).

The k_{600} values were converted to k values using the equation from Bartosiewicz et al. (2015):

$$k = k_{600}(Sc/600)^c,$$

where k is the gas transfer coefficient, c equals -0.67 according to Guérin et al. (2007), and Sc is calculated following Wanninkhof (2014). While there are acknowledged uncertainties associated with calculating k from wind-speed models, we aimed to provide an estimate of diffusive emissions from high-elevation lakes that is lacking in the literature. However, these estimated diffusive fluxes were not included in further statistical analyses.

2.3.4 Nutrients and organic matter

For nutrient measurements, water collected in the lakes was filtered (0.22 μm) and analyzed for NH_4^+, NO_2^-, NO_3^-, and PO_4^{3-}. Ammonium and NO_2^- were analyzed using a Trilogy Laboratory Fluorometer (Turner Designs; San Jose, CA, USA) with NH_4^+ and NO_2^- modules using the fluorescent method of Holmes et al. (1999) and the sulfanilamide coloration method (APHA 1998), respectively. Samples were frozen at -20 °C after collection and stored for no longer than two months (Chapman & Mostert, 1990). Standards ranged from 31 to 186 nM for NH_4^+ and 0 to 10,000 nM for NO_2^-, and standard curve r^2 values ranged from 0.997 to 0.999 for different runs. Nitrate and phosphate were analyzed using flow injection analysis on a QuikChem 8000 (Zellweger Analytics; Concord, ON, CA) at the University of California, Santa Barbara, Marine Sciences Institute Analytical Laboratory (standard curves $r^2 = 0.996$ and $r^2 = 0.997$, respectively).

Samples for DOC were only collected in 2017. Water was filtered through a GFF Whatman filter and collected in glass vials what had been combusted previously in a muffle furnace. Samples for DOC were acidified to pH 2 with 2 M HCl and analyzed in a TOC analyzer (Shimadzu TOC-Vcsh Total Organic Carbon Analyzer, Kyoto, Japan) at the Environmental Analytical Laboratory at University of California, Merced; standards ranged from 0 to 25 mg L^{-1}, with a standard curve $r^2 = 0.999$. Samples for dissolved organic matter (DOM) absorbance were kept in the dark at 4 °C for no longer than 5 days until analyzed in a Genesys 10 UV spectrophotometer for absorption at 254 nm (Thermo Scientific; Madison, WI, USA). The specific UV absorbance at 254 nm ($SUVA_{254nm}$) was calculated by dividing measured 254 nm absorbance per m of path length values by the DOC concentration of each sample. These $SUVA_{254nm}$ values have been used as indices of DOC aromaticity (e.g.,Weishaar et al., 2003).

Apparent oxygen utilization (AOU) was calculated from the following equation:

$$AOU = O_{2,sat} - O_2,$$

where $O_{2,sat}$ is the saturation oxygen concentration dependent on temperature and salinity and O_2 is the observed oxygen concentration.

2.3.5 Statistical analyses

We tested relationships between CH_4 concentrations in lake surface water and the following individual environmental variables using linear regression: T, DO, NO_2^-, NO_3^-, NH_4^+, PO_4^{3-}, ratios of dissolved inorganic nitrogen to dissolved inorganic phosphorus (DIN:DIP), DOC, $SUVA_{254nm}$, and elevation. We also used these same variables in standard multiple-linear regression analyses to predict CH_4 concentrations. Predictor variables and multi-linear models were selected based upon adjusted R^2 values, Aikake Information Criteria (AIC) of goodness of fit, and model significance. A priori significance level was defined as $\alpha<0.05$. We ran multi-linear regressions for the pooled data as well as for mid-elevation (Lukens, Upper and Lower Cathedral Lakes) and upper-elevation (Lower and Upper Gaylor Lakes) lakes separately. Data were assessed to ensure they met the assumptions of regression (independence of observations, homoscedasticity, and normality of residuals). Methane concentrations were natural-log-transformed due to non-normality of residuals and heteroscedasticity, but the predictor variables met all linear regression assumptions previously mentioned. All statistical analyses were performed using IBM SPSS Statistics for Macintosh, Version 25.0. Graphs were developed with Plotly Technologies Inc. Chart Studio (Cambridge, MA, USA) and SPSS (Chicago, IL, USA).

2.4 Results

2.4.1 Methane concentrations and diffusive fluxes within and across lakes

Methane concentrations and fluxes in lakes are often measured at single time points, even though they may have a highly dynamic nature. In our study, CH_4 concentrations showed large variations across lakes and over time, ranging from 16 to 3679 nM (Fig. 2.2a). All of these values were supersaturated, indicating that all lakes were net sources of CH_4 to the atmosphere at all times (Table 2.1). Within individual lakes, the highest and most variable CH_4 concentrations were found in Lukens and Lower Gaylor Lakes. Lukens is located at the lowest elevation and generally showed the highest CH_4 concentrations—although the single highest value was observed in Lower Gaylor, the majority of concentrations >500 nM occurred in Lukens Lake (Fig. 2.2a). Methane concentrations were highest in Lukens Lake in August in both years. In 2016, CH_4 concentrations showed a clear peak, while in 2017, CH_4 ranged from 1000 to1500 nM before declining later in September to a November minimum. Lower Gaylor had highly variable CH_4 concentrations in 2017, with an abnormally high value of 3679 nM in July 2017. Unlike the rest of the lakes, Lower Gaylor CH_4 concentrations were also significantly elevated at the end of the sampling season in late September and early October 2017, when temperatures were lowest. However, during 2016, CH_4 concentrations and patterns in Lower Gaylor Lake were more similar to the other lakes—values were <500 nM, with the highest values in summer. Methane concentrations were typically confined to a narrow range within both Cathedral Lakes, ranging from 132 to 354 nM in Lower Cathedral and from 129 to 356 nM in Upper Cathedral, with the highest concentrations occurring in July through August for both lakes. Upper Gaylor is located at the highest elevation and presented the lowest surface CH_4 concentrations, ranging from 16 to 280 nM. In both 2016 and 2017, CH_4 concentrations were the highest earlier on the sampling season. Similar to the other lakes, CH_4 concentrations reached a minimum at the end of the sampling season. All lakes therefore showed significant temporal variation in dissolved CH_4 concentrations.

Diffusive CH_4 fluxes ranged from 0.007 to 2.3 mmol m^{-2} day^{-1} (Fig. 2.2b, Table 2.1). Lukens Lake exhibited higher diffusive fluxes during 2016 than 2017 (Fig. 2.2b, Table 2.1), and diffusive fluxes were generally higher in August through September (with the exception of July 2017), while Cathedral and Upper Gaylor lakes observed fluxes of >0.2 mmol m^{-2} day^{-1} during the whole sampling season for both 2016 and 2017. Lower Gaylor diffusive fluxes were low overall in 2016 (0.03-0.3 mmol m^{-2} day^{-1}), but higher and more variable in 2017, when we observed the highest diffusive flux in July (2.3 mmol m^{-2} day^{-1}) followed by the lowest in August (0.08 mmol m^{-2} day^{-1}).

2.4.2 Seasonal and elevational variation in methane and temperature

Along with variation in CH_4, temperature showed expected seasonal and elevational variation within and across lakes. We observed similar seasonal trends in all lakes, where peak temperatures occurred in late July and early August, and temperatures were lowest at the end of the sampling season in October through November for both 2016 and 2017 (Fig. 2.2c). Within individual lakes, Lukens Lake is located at the lowest elevation (2489 m) and experienced the highest temperatures, ranging from 11.6 ˚C to 22.4 ˚C. At higher elevations, Lower Cathedral reached the highest temperature on July 2016 at 21.0˚C, and the lowest on October 2017 at 10.9 ˚C. Upper Cathedral had a similar temperature range (11.6-20.5 ˚C) and timing of peak values. The two lakes at the highest elevations presented the lowest temperatures at the beginning of the sampling season during ice thaw, and again at the end of sampling season in October, when temperatures declined to 7.9 ˚C in Lower Gaylor and 7.7 ˚C in Upper Gaylor. Highest temperatures in these lakes occurred in July 2016: 18.8 ˚C for Lower Gaylor and 15.5 ˚C for Upper Gaylor. Temperature seasonal trends were consistent for both 2016 and 2017; these temporal variations emphasize the large temperature range that high-elevation lakes display and the effect of elevation on their average temperatures.

Given coincident seasonal variations in CH_4 concentrations and temperature, consistent differences between lakes at different elevations, and previous work demonstrating temperature effects on CH_4 emissions, we analyzed potential relationships between temperature and CH_4 concentrations (Fig. 2.3a). Within individual lakes, CH_4 concentrations correlated with seasonal temperature variations in lake water in Lukens ($r^2 = 0.65$, $p<0.005$, n = 12), Lower Cathedral ($r^2 = 0.53$, $p<0.05$, n = 8) and Upper Cathedral Lake ($r^2 = 0.81$, $p<0.005$, n = 8). Methane concentrations were not significantly related to temperature in Upper Gaylor Lake, and were inversely related to temperature in Lower Gaylor Lake ($r^2 = 0.43$, $p<0.05$, n = 11). Data pooled across all lakes showed no significant correlation between CH_4 concentrations and temperature, likely due to the different patterns observed in the individual lakes. In particular, lower elevation lakes with more substantial temperature variation showed stronger correspondence between temperature and CH_4. We therefore analyzed relationships with elevation and found that elevation was the most significant factor determining CH_4 concentrations in the water—overall, the lower the elevation, the higher the CH_4 concentration in the lake ($r^2 = 0.39$, $p<0.005$, n = 49). Temporal variations within individual lakes are superimposed on this overall pattern.

2.4.3 Organic matter production and composition

To examine potential relationships between CH_4 concentrations and organic C dynamics, we measured DOC and SUVA in 2017, and DO in 2016 and 2017. DOC followed an elevational trend (Table 2.1); Lukens Lake presented the highest DOC values (2.54 - 3.74 mg L^{-1}), followed by Lower and Upper Cathedral Lakes (1.36 – 2.78 mg L^{-1} and 1.65 – 1.97 mg L^{-1}, respectively), and Lower Gaylor presented the lowest values most of the season (1.29 – 2.58 mg L^{-1}). We observed a DOC maximum in August and a minimum in October for all lakes in 2017. Higher CH_4 concentrations correlated with higher DOC concentrations in lake water for the measurements taken from all lakes (r^2 = 0.30, p<0.05, n = 16; Fig. 2.7a). Methane concentrations were also significantly correlated with $SUVA_{254nm}$ (r^2 = 0.23, p<0.05, n = 16; Fig. 2.7b).

We also measured DO, as it (1) is affected by changes in temperature, (2) can integrate changes in production and consumption of organic matter, and (3) affects the redox favorability of CH_4 production and oxidation in sediments and water column. During both 2016 and 2017, DO increased over the summer and fall with the lowest values in July and the highest in October (Fig. 2.4a). Dissolved oxygen ranged from 6.0 to 8.0 mg L^{-1} in Lukens Lake, 6.0 to 7.8 mg L^{-1} in Lower Cathedral Lake, and 5.0 to 7.7 mg L^{-1} in Upper Cathedral Lake. Gaylor Lakes, located at the highest elevation, presented the highest DO, with Lower Gaylor ranging from 5.0 to 8.7 mg L^{-1} and Upper Gaylor ranging from 6.0 to 8.9 mg L^{-1}. Methane concentrations were inversely correlated with DO in Lukens Lake (r^2 = 0.69, p<0.005, n = 11), while correlations for the other lakes individually or using pooled data across all five lakes were not significant. Given that variations in temperature likely affect observed DO concentrations, we calculated apparent oxygen utilization (AOU) from the difference between DO values expected in equilibrium with the atmosphere at different temperatures versus those observed. Apparent oxygen utilization showed consistent seasonal trends in most of the lakes (Figure 2.4b). However, CH_4 concentrations were not significantly related to AOU.

2.4.4 Inorganic nutrients dynamics

We measured three forms of dissolved inorganic nitrogen (DIN; ammonium, nitrite, and nitrate), as well as dissolved inorganic phosphorus (DIP; phosphate). High-elevation lakes are typically nutrient depleted (Sickman et al. 2003; Moser et al. 2019), and the lakes in this study are no exception, with low concentrations of all measured inorganic nutrients (Fig. 2.5).

Ammonium concentrations were low and variable (0.18 to 2.83 μM, with the majority of NH_4^+ concentrations <1.5 μM), but in contrast to CH_4, NH_4^+ concentrations did not display a seasonal trend and CH_4 was not significantly related to NH_4^+ (Fig. 2.5a). Both NO_2^- and NO_3^- concentrations were also low over the period studied, typically ranging from 0 to 1 μM (Fig. 2.5b and 2.5c). Nitrite trends in the lakes differed from

year to year. In 2016, overall variation was higher, and there were no discernible seasonal trends; in 2017, all NO_2^- concentrations were uniformly <0.3 μM, and we observed a seasonal trend for Lukens, Lower and Upper Cathedral Lakes. Methane concentrations were statistically significantly correlated to NO_2^- in lake water in Lukens (r^2 = 0.41 p<0.05, n = 12) and Upper Cathedral (r^2 = 0.52, p<0.05, n= 8). Methane concentrations in Lower and Upper Cathedral Lakes were also significantly related to NO_3^- in the water (r^2 = 0.64, p<0.05, n = 7 for Lower Cathedral and r^2 = 0.67, p<0.05, n = 8 for Upper Cathedral). For both NO_2^- and NO_3^-, there were no other significant relationships for individual lakes or the pooled lake data. Phosphate concentrations remained below 0.8 μM in these oligotrophic mountain lakes (Fig. 2.5d). Phosphate concentrations were more variable in 2016 (0.1 to 0.8 μM) compared to 2017 (0.0 to 0.2 μM), especially for Lukens and Lower and Upper Gaylor Lakes. However, none of the lakes showed discernible seasonal trends over the summer (although PO_4^{3-} tended to be lowest at the end of the sampling season in October), and CH_4 was not significantly related to PO_4^{3-}.

The DIN:DIP ratios were typically low, ranging from 3 to 18 for 2016 and 2 to 25 in 2017 (Fig. 2.5e). Low DIN:DIP ratios indicate that the lakes studied are mainly N limited (DIN:DIP < 10), with fewer cases of DIN:DIP ratios indicative of co-limitation (10 - 17), or P limitation (DIN:DIP > 17; Morris and Lewis 1988; Nürnberg and Shaw 1998). Upper Cathedral CH_4 concentrations showed a negative correlation with DIN:DIP ratio (r^2 = 0.64, p<0.05, n = 8), while the other lakes individually and overall nutrient dataset were not significantly related to CH_4 concentrations.

2.4.5 Multi-linear model for methane concentrations in high-elevation lakes

Given the seasonal and elevation-related patterns observed in high elevation lakes, we tested for statistically significant relationships between CH_4 and potential explanatory variables using multiple linear regression. Pooling all lakes together showed that the only significant predictor variable across all lakes was elevation (Table 2.2); however, individual lakes displayed strong temperature responses, especially at lower elevations. As a result, we classified lakes into two elevational bands (mid- and upper elevation) to better understand relationships at different elevations (Table 2.2). The multilinear regression in the mid-elevation band showed that CH_4 concentrations in lake water were significantly correlated to temperature, elevation, and NO_2^-; in the upper-elevation lake band, CH_4 concentrations were significantly related to elevation and DIN:DIP ratios. Methane concentration in individual lake surface water and in the pooled lake data was consistently related to elevation, as well as in the elevation band regressions. Contrary to our hypothesis, CH_4 concentrations in mid-elevation lakes, but not upper-elevation lakes, were positively correlated to temperature over the growing season.

2.5 Discussion

2.5.1 Seasonal variation in methane

Our findings have multiple implications for our understanding of CH_4 biogeochemistry in freshwater ecosystems. First and most fundamentally, significant seasonal variability was clearly evident in CH_4 concentrations in Sierra Nevada lakes, as highest CH_4 concentrations were observed in the warmest months, and lowest CH_4 concentrations were typically observed at the end of the sampling season before winter. Field site accessibility prevented taking measurements during the winter and spring seasons, but Greene et al. (2014) and Jammet et al. (2015) showed that there can be significant CH_4 release during ice-off due to the build-up of CH_4 under ice throughout winter and spring. While this phenomenon was not captured here and could be significant, it underlines our over-arching finding that dissolved CH_4 can be highly variable over time.

Second, our data indicate that single time point measurements of CH_4 concentrations in lakes do not adequately reflect overall seasonal CH_4 diffusive flux, as CH_4 concentrations varied 3- to 73-fold over time in our study. Previous seasonal CH_4 measurements have been conducted in only a handful of lakes worldwide and also show significant variation. For example, Casper et al. (2009) observed a seasonal cycle in a single lake in Germany, where CH_4 lake concentrations and fluxes increased in the summer and decreased by winter. Similarly, Palma-Silva et al. (2013) detected higher CH_4 concentrations in one oligotrophic and one eutrophic lake in Brazil when higher temperatures were observed—a finding shared for a single shallow pond in Sweden (Natchimuthu et al. 2014), and for a single subtropical lake in China (Xing et al. 2005). Contrarily, Martinez-Cruz et al. (2015) found the opposite pattern for thirty Alaskan Lakes, where CH_4 concentrations were on average lower during the summer and higher during the wintertime due to changes in lake ice cover. Taken together, these limited data indicate that seasonal CH_4 variations can be significant, and our results provide additional context from five contrasting, high-elevation lakes in the Sierra Nevada.

Third, changes in the length of the growing season will likely increase overall CH_4 diffusive flux if periods of high CH_4 concentrations in lake water expand in time. This may be especially relevant for high-elevation lakes, where warmer air temperatures will increase the lake ice-free period, increase water temperature, and potentially increase organic matter and nutrient inputs from the surrounding watershed—at least in the short-term (Moser et al. 2019; Sadro et al. 2019). In line with this idea, our results showed that CH_4 concentrations in high-elevation lakes were most strongly correlated with elevation, with higher average CH_4 concentrations at lower elevations. Elevation can be a proxy for temperature, as lower elevations showed the lowest mean temperatures. If high-elevation lakes shift to resemble lower-elevation lakes, the strong correlation with elevation suggests increased CH_4 concentrations. However, both organic C concentrations and composition, as well as nutrient concentrations, may also vary with elevation. In general, lakes at higher

elevation tend to be more oligotrophic, as allochthonous sources of C and nutrients become more scarce in alpine and sub-alpine regions (Urmy & Warren 2019). We therefore evaluated organic matter and nutrients as potentially relevant factors for CH_4 cycling in lakes that are also elevation dependent.

2.5.2 Relationships between methane, organic matter production and composition, and dissolved nutrients

The quality and quantity of organic matter can affect methanogenesis in lakes because organic matter provides substrates for CH_4 production, and affects oxygen availability in sediments due to heterotrophic aerobic respiration (Tranvik et al. 2009; Grasset et al. 2018; Sepulveda-Jauregui et al. 2018). We found that patterns in DOC and $SUVA_{254nm}$ were consistent with lake elevation and watershed characteristics (Table 2.1). In particular, Lukens Lake is located at the lowest elevation with a surrounding meadow, and likely has a larger input of allochthonous C, while Lower and Upper Cathedral Lakes are at higher elevation where allochthonous C would be relatively lower. Lower and Upper Gaylor Lakes are located above the tree line where DOC levels tend to be lower (Moser et al., 2019). Consistent with this, DOC concentrations were the highest at the lowest elevation and decreased with increases in elevation (Table 2.1). The specific UV absorbance at 254 nm is a useful measure to perceive changes in organic C over the growing season. Low $SUVA_{254nm}$ is indicative of overall low molecular weight (Chowdhury, 2013) and low percent aromaticity (Weishaar et al., 2003). Allochthonous sources of organic C are often complex molecules (aliphatic polymers, humic substances) that are mainly degraded under aerobic conditions, whereas autochthonous sources can be mineralized under both aerobic and anaerobic conditions (Zehnder & Svensson 1986; Hulthe et al. 1998; Bastviken et al. 2004). In our study, the inverse correlation between CH_4 concentrations and $SUVA_{254nm}$ values (Fig. 2.7) suggests that simple C molecules favor enhanced CH_4 production.

High-elevation lakes are also often oligotrophic, such that changes in nutrient concentrations can affect the overall ecology of the lake. Higher nutrient input to lakes can alter lake community structure and enhance primary productivity and CH_4 production (Tranvik et al. 2009; West et al. 2016; Reay et al. 2018). Overall, we found low concentrations of all dissolved inorganic nutrients—consistent with oligotrophic conditions prevalent in high-elevation lakes. Although both nitrogen (N) and phosphorus (P) may be limiting nutrients in freshwater ecosystems (Elser et al., 2007), P availability is particularly relevant to the CH_4 paradox (i.e., the observation of consistent supersaturation of CH_4 in freshwater and marine oxic surface waters; Karl et al. 2008; Tang et al. 2014). Several studies have proposed that biological mechanisms other than traditional methanogenesis produce CH_4 (Grossart et al. 2011; Bogard et al. 2014; Tang et al. 2016; Bižić et al., 2020) and one mechanism that may be particularly important in oligotrophic ecosystems is the demethylation of methyl phosphonate. This can be performed by multiple groups of bacteria using C-P lyase genes during the degradation of DOM (Repeta et al. 2016; Yao et al. 2016; Wang et al. 2017), and has been observed in both freshwater (Yao et al. 2016; Wang et al.

2017) and marine (Karl et al. 2008; Metcalf et al. 2012; Carini et al. 2014; Repeta et al. 2016) ecosystems.

In our study, we found low but detectable PO_4^{3-} concentrations through most of the ice-free season in most lakes. This result is consistent with a long-term study of Emerald Lake in the Sierra Nevada (Sickman et al. 2003). We focused on inorganic nutrient concentrations because previous work has shown that ecological changes associated with nutrient enrichment can affect CH_4 fluxes, and changes in bioavailable P could affect CH_4 production via methyl phosphonate breakdown. In particular, we might expect that low P availability overall, or in comparison to N, might result in increased microbial methylphosphonate breakdown, and therefore increased CH_4 production. However, we did not observe significant relationships between CH_4 concentrations and DIP.

Instead, CH_4 was related to dissolved nitrite, nitrate, and DIN:DIP ratios within some individual lakes and in multiple linear regression (Fig. 2.6, Table 2.2). While CH_4 concentrations were not as consistently related to inorganic nutrients as they were to temperature and elevation, they still displayed significant correlations in some of the high-elevations lakes studied here (Fig. 2.6). Higher elevation lakes exhibit lower nutrient and DOC concentrations than mid-elevations lakes (Moser et al. 2019; Urmy & Warren 2019); in this area, microbial activities may be constrained by nutrient concentrations. Previous studies have found that alpine ecosystems are highly sensitive to modest N deposition (Baron et al. 2000; Wolfe et al. 2003; Vinebrooke et al. 2014). Consistently, lakes in this study were likely N-depleted for the majority of the season, which may explain why CH_4 concentrations were predicted to be higher when N was more available either in the NO_2^- form or as a higher DIN:DIP ratio.

2.5.3 Methane dynamics in relation to elevation and temperature

We found significant relationships between CH_4 concentrations and elevation and temperature. Highest CH_4 concentrations were observed at the lowest elevation lake and decreased with elevation. While associated with elevation, temperature was also correlated with CH_4 concentrations measured over time in the mid-elevation lakes (<3000 m, Lukens, Upper and Lower Cathedral Lakes). In our study, lakes located at different elevations with different mean temperatures showed distinct seasonal patterns in temperature and CH_4 concentrations. Likewise, lakes sampled in earlier work also varied in the strength of CH_4-temperature relationships (Xing et al. 2005; Casper et al. 2009; Palma-Silva et al. 2013; Natchimuthu et al. 2014), but showed that temperature increases have overall a corresponding positive response on lake CH_4 concentrations (Natchimuthu et al. 2014; Marotta et al. 2014; Yvon-Durocher et al. 2014; Rasilo et al. 2015). Our data provide additional evidence of positive temperature-CH_4 relationships in several lakes.

Collectively, these observations lend support to the hypothesis that CH_4 concentrations are typically closely related to temperature, but other factors that vary seasonally or with elevation may also be important in regulating CH_4 concentrations

in lakes. In particular, CH_4 concentrations were correlated with temperature, elevation, and NO_2^- in mid-elevation lakes. In contrast, the lack of a temperature response in the upper-elevation lakes indicates that CH_4 concentrations may be affected by other factors. For example, nutrient availability regulates lake productivity, and nutrient increases have been correlated with enhanced CH_4 emissions (Palma-Silva et al. 2013; West et al. 2016). Disentangling the relative influence of temperature, nutrients, and C in high elevation lakes may be achieved through additional experimental work, as our measurements indicate that all of these factors can be significantly related to CH_4 concentrations.

Methane ebullitive fluxes were not measured in this study but can be a major contribution to total lake CH_4 emissions, especially in shallow lakes (Bastviken et al. 2004; DelSontro et al. 2016). Lake CH_4 ebullitive emissions have been found to be significantly sensitive to increases in temperature and changes in nutrient concentrations (DelSontro et al., 2016) Aben et al. 2017; Davidson et al. 2018) and deserve further research. Combining these data from five Sierran lakes with additional observations (including ebullitive fluxes) from other temperate montane lakes may result in the development of robust, multivariate predictive models to accurately predict CH_4 concentrations and emissions over space and time as the climate continues to warm. Concentrations of CH_4 in high elevation lakes are notably high and variable, and are indicative of dynamic CH_4 cycling. Our study provides additional fundamental information on freshwater CH_4 biogeochemistry in montane lakes; these data should be useful in the development of predictive models of CH_4 fluxes from freshwater ecosystems under this current period of rapid global change.

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2.7 Tables

Table 2.1 Summary of descriptive statistics of environmental parameters measured from early July to late October 2016 and 2017 for five temperate montane lakes in the Sierra Nevada, California, USA.

	All				Lukens				Lower Cathedral				Upper Cathedral				Lower Gaylor				Upper Gaylor			
	N	Min	Max	Mean	N	Min	Max	Mean	N	Min	Max	Mean	N	Min	Max	Mean	N	Min	Max	Mean	N	Min	Max	Mean
Methane (nM)	49	16	3679	553	12	562	2839	1226	8	132	354	216	8	129	356	251	12	50	3679	652	9	16	280	93
Methane (% saturation)	49	461	104,951	17,758	12	16,800	93,800	41,300	8	4000	11,300	7000	8	4000	11,300	8400	12	1600	104,951	18,800	9	461	8500	2800
Diffusive methane flux (mmol m^{-2} day^{-1})	47	0.007	2.3	0.3	12	0.1	1.6	0.66	8	0.05	0.2	0.11	8	0.06	0.2	0.13	11	0.03	2.3	0.4	8	0.007	0.2	0.05
Temperature (°C)	47	7.7	22.4	15.6	12	11.2	22.4	17.2	8	10.9	21	16.7	8	11.6	20.5	17.4	11	7.9	18.8	13.8	8	7.7	15.5	12.8
Dissolved oxygen (mgL^{-1})	43	5	8.9	7.3	11	6	7.9	7.2	8	6	7.8	7	7	5	7.7	6.9	10	5	8.7	7.6	7	6	8.9	7.7
Dissolved oxygen (% saturation)	42	60	81.5	74.6	11	65	81.5	75.3	8	70	80	74.8	7	60	76.6	71.3	9	60	78.1	76.4	6	70	80	74
Apparent oxygen utilization (mgL^{-1})	43	-1.43	1.37	-0.3	11	-0.76	0.99	-0.04	8	-0.49	0.26	-0.15	7	-0.73	1.37	-0.13	10	-1.43	1.3	-0.48	7	-1.17	0.93	-0.47
Nitrite (µM)	48	0	0.7	0.2	12	0	0.7	0.2	7	0.0	0.44	0.2	8	0	0.3	0.2	12	0	0.4	0.2	9	0	0.4	0.2
Nitrate (µM)	46	0	1.9	0.4	12	0	0.8	0.3	7	0	1.34	0.5	8	0.1	0.4	0.3	12	0	1.9	0.6	7	0.2	0.9	0.4
Ammonium (µM)	48	0.3	2.8	0.9	12	0.5	1.9	0.7	7	0.3	2.4	0.8	8	0.3	1.2	0.7	12	0.5	2.8	1.3	9	0.3	1.9	0.8
Phosphate (µM)	46	0	0.8	0.2	12	0.1	0.4	0.2	7	0.1	0.2	0.1	8	0	0.2	0.1	12	0.1	0.8	0.3	7	0.1	0.4	0.2
Dissolved inorganic nitrogen :dissolved inorganic phosphorus	46	2.5	25.4	8.9	12	2.5	17.7	6.7	7	4.2	24.7	10.1	8	6.4	20.3	10.9	12	3.5	25.4	9.5	7	3.9	13.4	7.8
Dissolved organic carbon (mgL^{-1})	16	1	10.7	2.5	4	2.5	10.7	5	4	1.4	2.78	1.9	3	1.65	1.97	1.8	4	1.3	2.6	1.7	1			1
Specific UV absorbance at 254 nm (L $mg–C^{-1}$ m^{-1})	16	0.5	2.7	1.6	4	0.5	1.9	1.5	4	1.2	2.36	1.9	3	1.8	2.3	2.3	4	0.9	1.3	1.1	1			1.5
Elevation (m)	49	2489	3185	2891.3				2489				2815				2905				3115				3185

Table 2.2 Multi-linear regression models for methane (CH_4) surface concentrations as a function of environmental parameters measured in five Sierra Nevada montane lakes. Abbreviations: NO_2^- = nitrate and DIN:DIP = dissolved inorganic nitrogen to dissolved inorganic phosphorus ratio.

Lakes	**Equation**	***R^2* adjusted**	***P*-value**	**n**
All Lakes	Predicted $\ln(CH_4) = 5.650 - 0.003(\text{Elevation})$	0.39	<0.005	49
Mid-elevation cluster (>3000 m) (Lukens, Lower and Upper Cathedral)	Predicted $\ln(CH_4) = 6.079 + 0.082(\text{Temperature}) - 0.004(\text{Elevation}) + 1.268(NO_2^-)$	0.90	<0.0005	27
Upper-elevation cluster (<3000 m) (Lower and Upper Gaylor)	Predicted $\ln(CH_4) = 5.128 - 0.022(\text{Elevation}) + 0.092\ (\text{DIN:DIP})$	0.48	<0.005	19

2.8 Figures

Lukens Lake	Lower Cathedral	Upper Cathedral	Lower Gaylor	Upper Gaylor
Elevation: 2489 m **Latitude:** 37.8598 **Longitude:** 119.6160 **Mean Temperature:** 17.2 °C **Mean CH_4 concentration:** 1226 nM **Depth:** 5 m	**Elevation:** 2815 m **Latitude:** 37.8450 **Longitude:** 119.4241 **Mean Temperature:** 16.7 °C **Mean CH_4 concentration:** 216 nM **Depth:** 11 m	**Elevation:** 2905 m **Latitude:** 37.8395 **Longitude:** 119.4154 **Mean Temperature:** 17.4 °C **Mean CH_4 concentration:** 251 nM **Depth:** 3 m	**Elevation:** 3115 m **Latitude:** 37.9093 **Longitude:** 119.2862 **Mean Temperature:** 13.8 °C **Mean CH_4 concentration:** 652 nM **Depth:** 13 m	**Elevation:** 3185 m **Latitude:** 37.9223 **Longitude:** 119.2673 **Mean Temperature:** 12.8 °C **Mean CH_4 concentration:** 93 nM **Depth:** 8 m
				

Figure 2.1 Morphology and environmental characteristics of five temperate montane lakes in the Sierra Nevada, California, USA. Depth represents the maximum depth observed in each lake. Mean surface temperature and methane concentration were calculated over the sampling period July-October 2016 and July-October 2017.

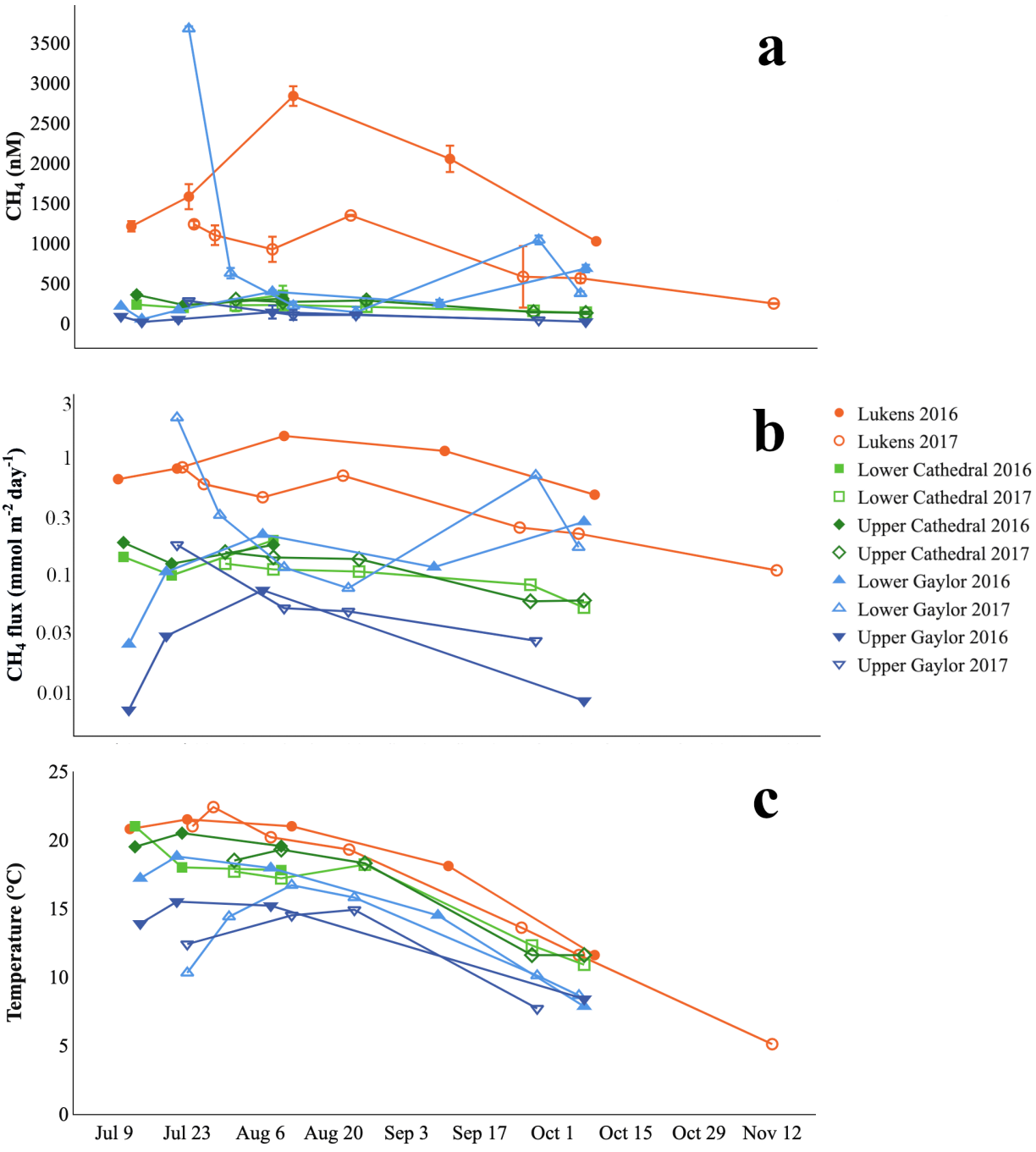

Figure 2.2 a) Dissolved methane concentrations (error bars represent standard deviation of triplicate water samples), b) methane effluxes (natural log transformed CH_4 diffusive flux), and c) water temperature in five temperate montane lakes in the Sierra Nevada, California, USA. Colored symbols denote different lakes sampled in 2016 (open symbols) and 2017 (closed symbols), with the date of sampling along the horizontal axis

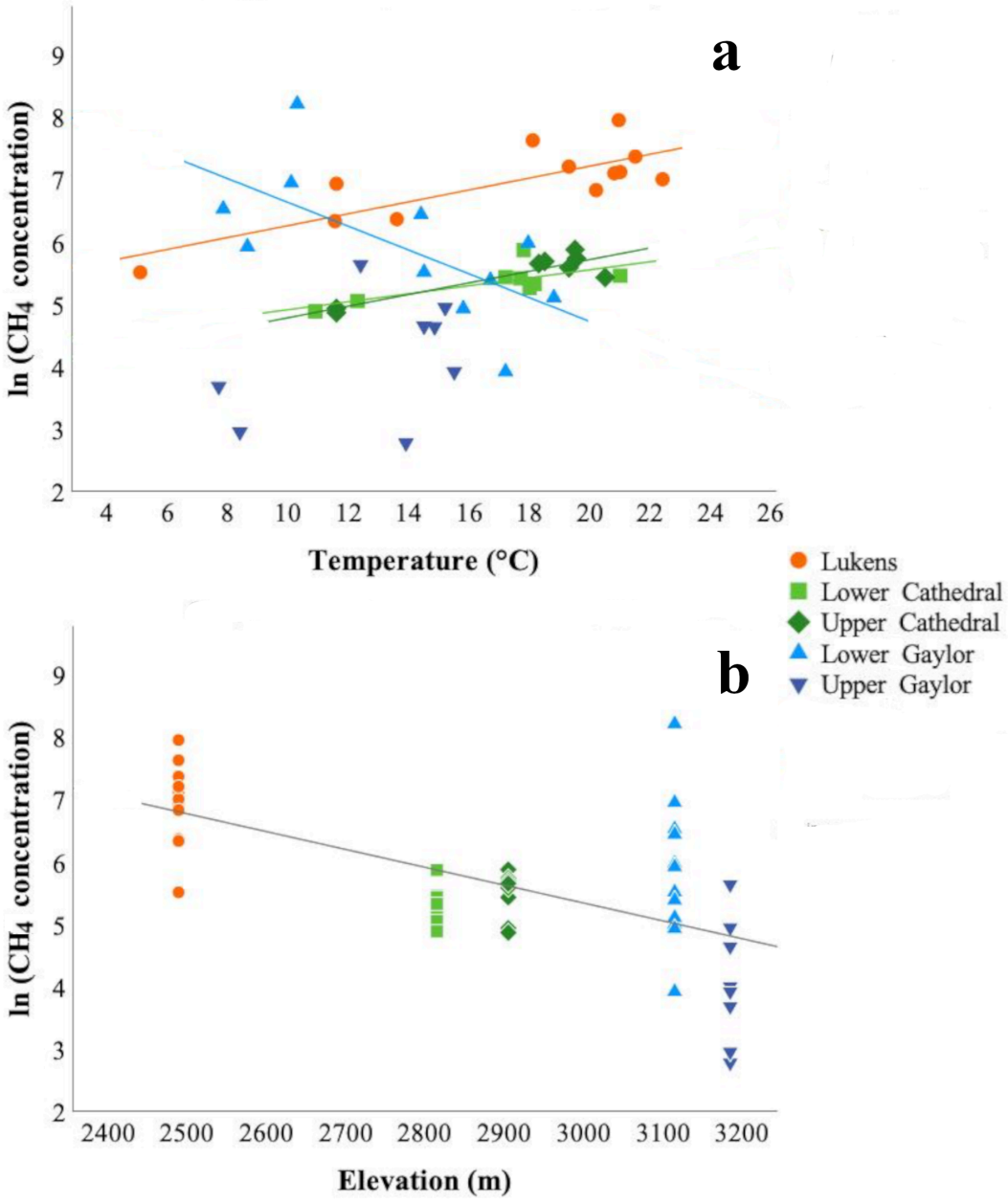

Figure 2.3 Dissolved methane concentrations in surface water (natural log transformed CH_4 concentrations) as function of a) temperature, and b) elevation for the five temperate montane lakes sampled in the Sierra Nevada, California, USA. Colored symbols denote different lakes sampled. Colored lines represent significant linear relationships for individual lakes (Lukens: ln (CH_4) = 5.31 + 0.1*Temperature; Lower Cathedral: ln (CH_4) = 4.27 +0.06*Temperature, Upper Cathedral: ln (CH_4) = 3.85 +0.09*Temperature; and Lower Gaylor: ln (CH_4) = 8.54 – 0.19*Temperature). Black line represents a significant linear relationship across all lakes (ln (CH_4) = 5.650 -0.003*Elevation)

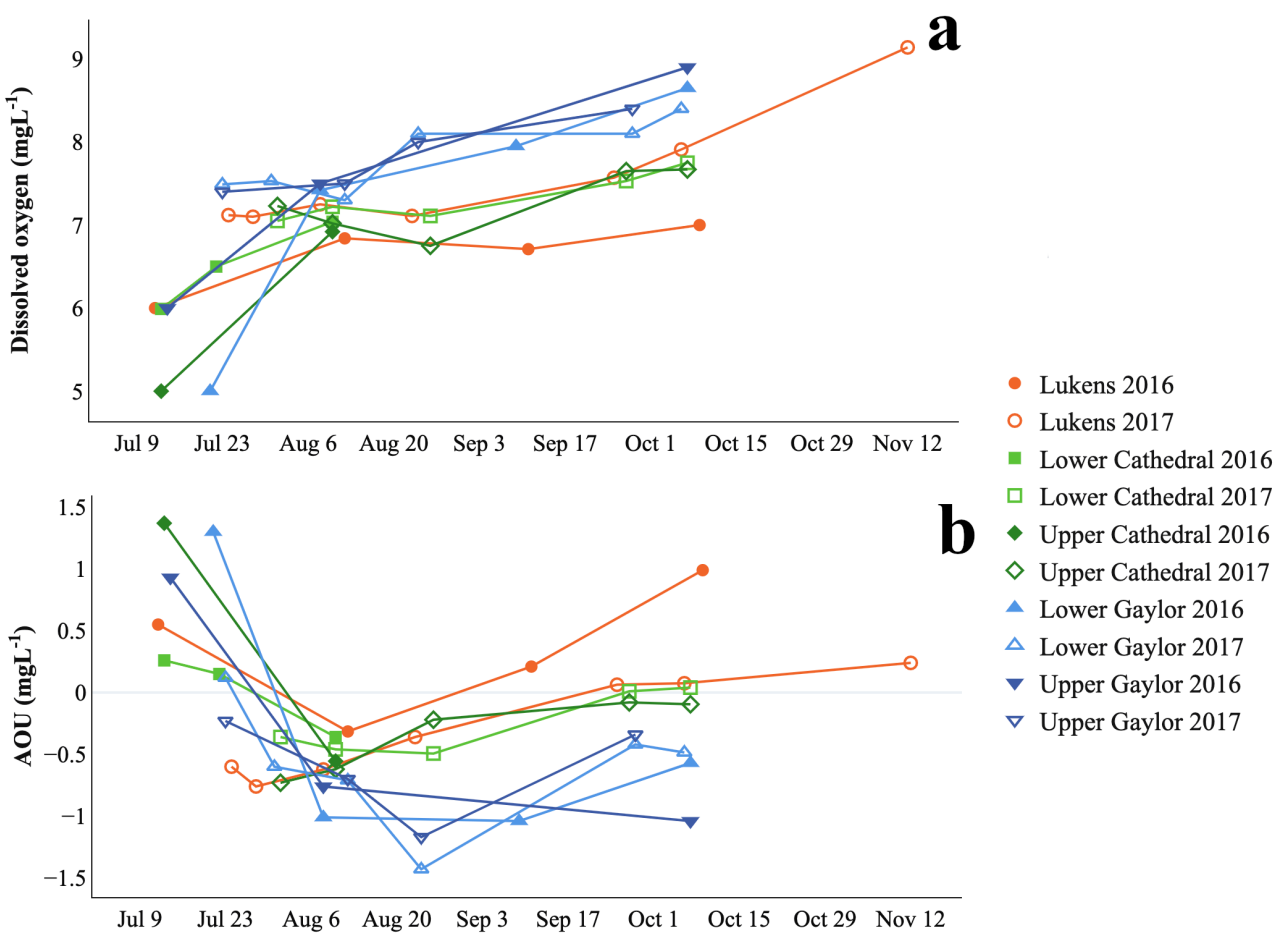

Figure 2.4 a) Dissolved oxygen concentrations and b) apparent oxygen utilization (AOU) of five temperate montane lakes in the Sierra Nevada, California, USA. Colored symbols denote different lakes sampled in 2016 (open symbols) and 2017 (closed symbols), with the date of sampling along the horizontal axis

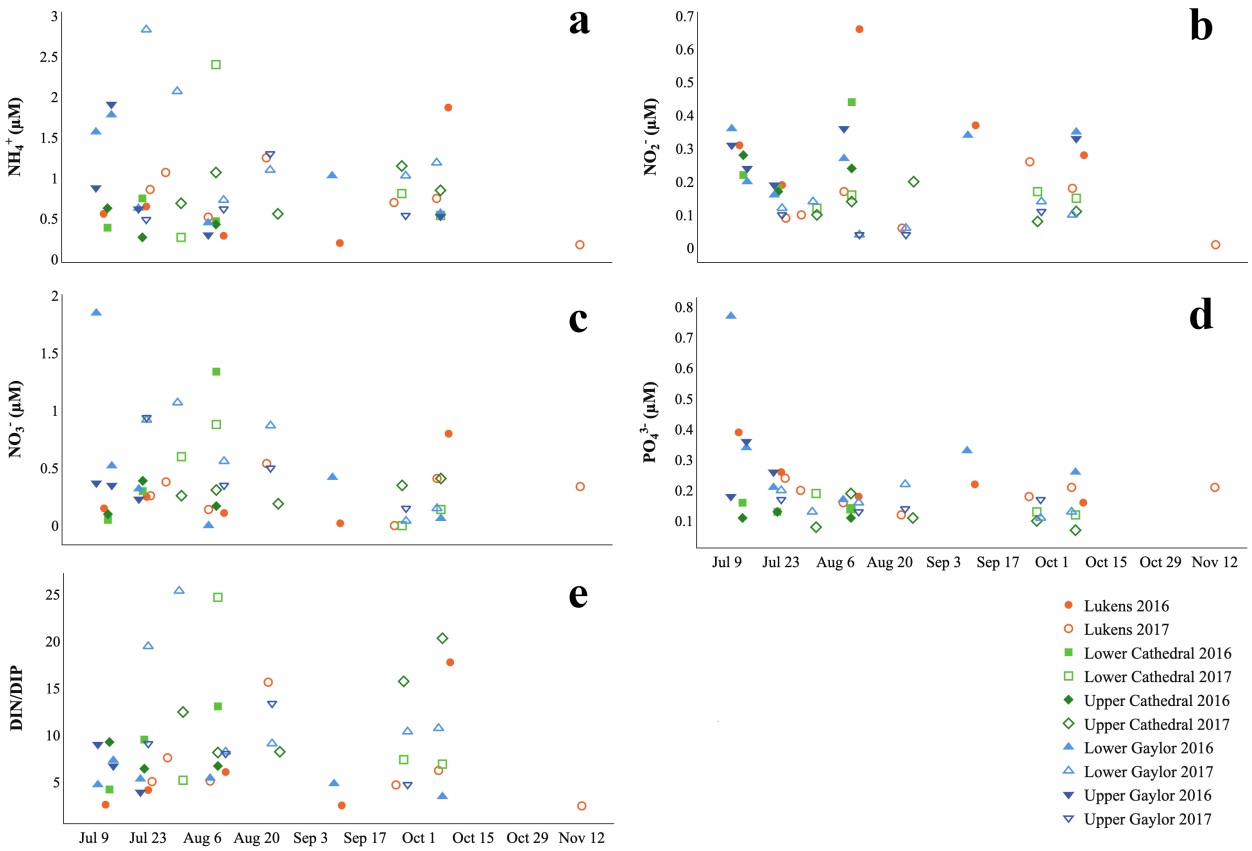

Figure 2.5 Inorganic nutrients concentrations: a) ammonium, b) nitrite, c) bitrate, d) phosphate, and e) dissolved inorganic nitrogen : dissolved inorganic phosphorus ratios for five temperate montane lakes in the Sierra Nevada, California, USA. Colored symbols denote different lakes sampled in 2016 (open symbols) and 2017 (closed symbols), with the date of sampling along the horizontal axis.

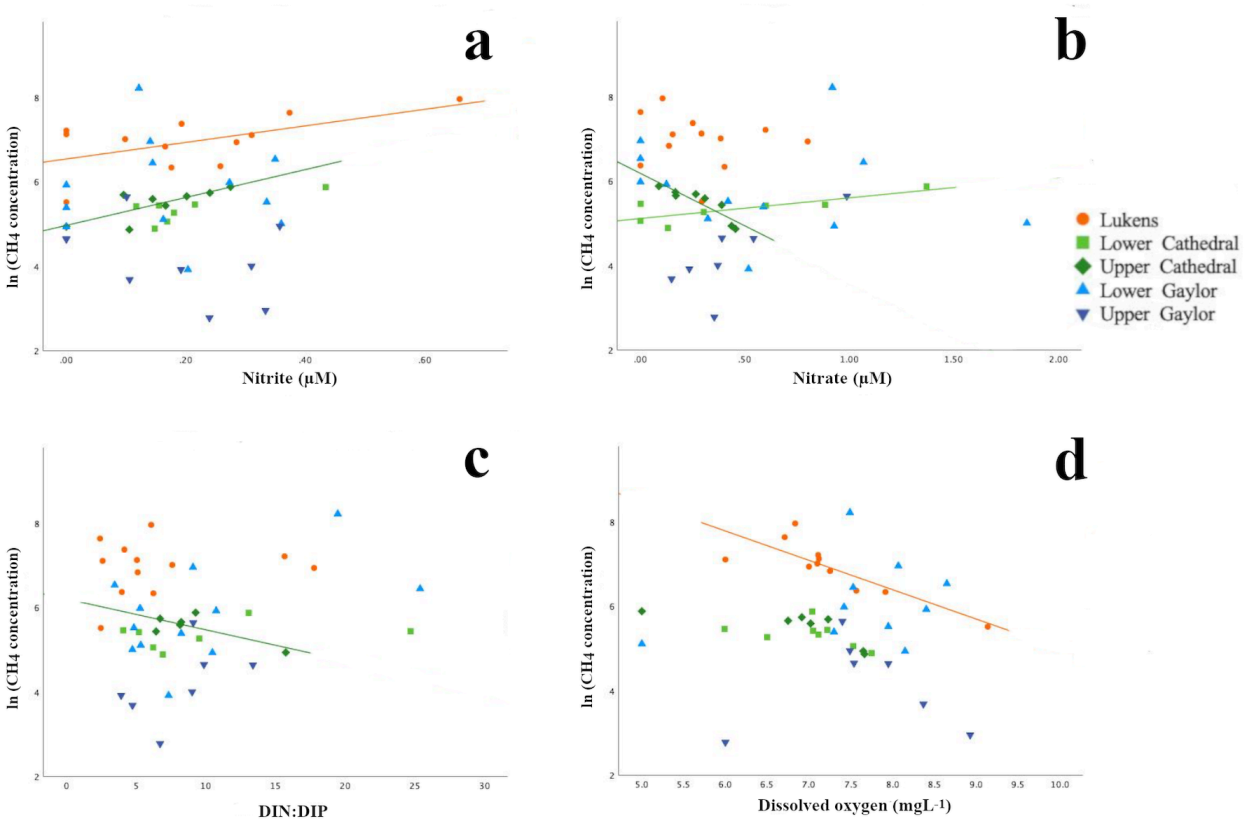

Figure 2.6 Dissolved methane concentrations in surface water (natural log transformed CH_4 concentrations) as function of a) nitrite concentrations, b) nitrate concentrations, c) Dissolved inorganic nitrogen : dissolved inorganic phosphorus ratios, and d) dissolved oxygen concentrations for the five temperate montane lakes sampled in the Sierra Nevada, California, USA. Colored symbols denote different lakes sampled. Colored lines represent significant linear relationships for individual lakes.

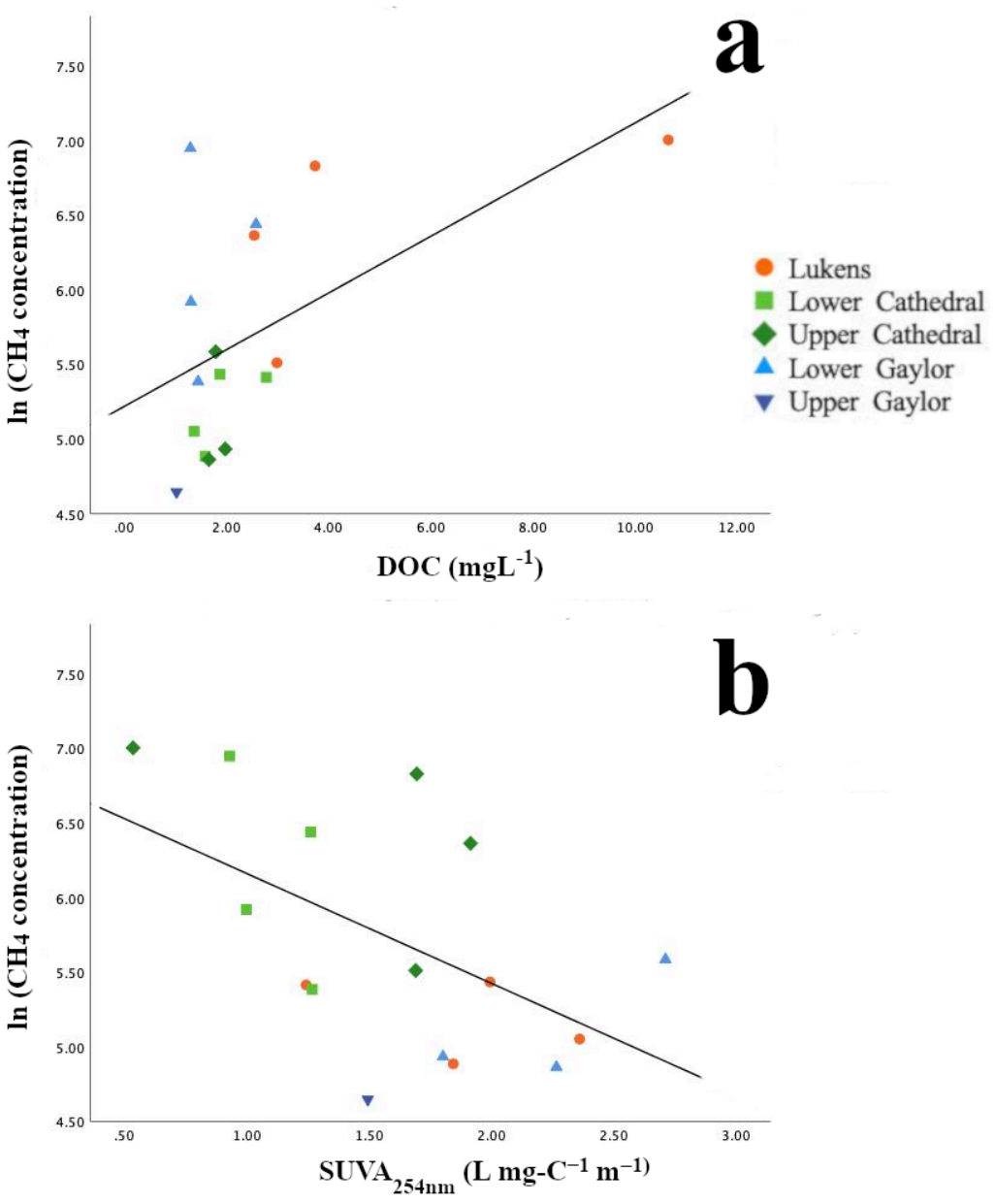

Figure 2.7 Dissolved methane concentrations in surface water (natural log transformed CH_4 concentrations) as function of a) dissolved organic carbon concentrations, and b) specific UV absorbance at 254 nm for the five temperate montane lakes sampled in the Sierra Nevada, California, USA. Colored symbols denote different lakes sampled. Black lines represent significant linear relationships.

3 Biogeochemical and multi-omic evidence for multiple mechanisms of paradoxical methane production freshwater lakes

3.1 Abstract

Aquatic ecosystems are globally significant sources of the greenhouse gas methane (CH_4) to the atmosphere. However, CH_4 is produced 'paradoxically' in oxygenated water via at least three mechanisms, fundamentally limiting our understanding of overall CH_4 production. Here we resolve these CH_4 production mechanisms through CH_4 measurements, $\delta^{13}CH_4$ analyses, 16S rRNA sequencing, and metagenomics/transcriptomics applied to freshwater incubation experiments with multiple time points and treatments (addition of a methanogenesis inhibitor, dark, high-light). We captured significant paradoxical CH_4 production, but show that methanogenesis was an unlikely CH_4 source. In contrast, abundant freshwater bacteria metabolized methylphosphonate—similar to observations in marine ecosystems. Metatranscriptomics and stable isotopic analyses applied to experimental treatments also identified a potential CH_4 production mechanism linked to (bacterio)chlorophyll metabolism by Cyanobacteria and especially Proteobacteria. Variability in these mechanisms across experiments indicates that multiple, widely-distributed bacterial groups and pathways can produce substantial quantities of CH_4 in aquatic ecosystems.

3.2 Introduction

Methane (CH_4) is a potent greenhouse gas with a global warming potential 28 times that of carbon dioxide (IPCC 2014). Atmospheric CH_4 concentrations have increased significantly due to anthropogenic activity and are an important component of climate change (IPCC 2014). However, these increases are superimposed on substantial natural variability. Of all natural CH_4 sources to the atmosphere, freshwater lakes are particularly important but poorly understood, with their estimated contribution ranging from 6-16% of all natural CH_4 emissions, despite accounting for only ~0.9% of the Earth's surface area (Bastviken et al. 2004). CH_4 emissions from lakes are conventionally viewed to be regulated by CH_4 production (occurring predominantly in anoxic sediments) and subsequent CH_4 oxidation in surface sediments and the water column (Bastviken 2009). However, oversaturation of CH_4 has been consistently observed in oxygenated waters of aquatic systems (Tang et al. 2016). This observation indicates that CH_4 is produced under oxic conditions, and that the rate of CH_4 production exceeds CH_4 oxidation. Since archaeal methanogenesis is an obligate anaerobic process (Hoehler et al., 2018), oxic CH_4 production is typically referred to as the "methane paradox," and has been observed in oceans (Karl et al. 2008; Damm et al. 2010), lakes (Grossart et al. 2011; Bogard et al. 2014; Tang et al. 2014), and aerobic wetland soils (Angle et al. 2017). Notably, paradoxical CH_4 production occurs near the surface, and so any produced CH_4 may readily flux to the atmosphere. Identifying which mechanisms produce CH_4 in oxygenated waters is

therefore essential for our understanding of methane fluxes and their contribution to climate change.

Although multiple mechanisms for paradoxical aerobic CH_4 production have been proposed, the degree to which these are active in freshwater lakes remains unknown. Initial studies suggested that CH_4 production under oxygenated conditions could be occurring in anoxic microsites in the water column—such as fecal pellets, detritus, and the gastrointestinal tracts of larger organisms such as fish or zooplankton (Oremland 1979; Traganza et al. 1979; Angelis & Lee 1994; Karl & Tilbrook 1994). Several studies have also demonstrated a correlation between phytoplankton or primary production and CH_4 production (Grossart et al. 2011; Bogard et al. 2014; Tang et al. 2014). While phytoplankton metabolites can be the source for methanogenesis in oxygenated water, Grossart et al. (2011) showed that methanogens can also live in the surface of phytoplankton and produce CH_4 in the phycosphere. Moreover, Bogard et al. (2014) and Donis et al. (2017) found that stable isotope signatures of CH_4 in their studies corresponded to those of acetoclastic methanogenesis. While methanogenesis is inhibited by oxygen, they hypothesize that several groups of methanogens have oxygen-tolerant or detoxifying pathways that could aid in methane production in the presence of oxygen. For example, Angle et al. (2017) characterized a methanogen candidate that possesses the enzymes to detoxify oxygen and produce CH_4 through the acetoclastic pathway in wetland soils.

In contrast, the current prevailing view of marine ecosystems is that methylphosphonate (MPn) is the main precursor of methane production under oxic conditions, particularly in phosphate-stressed ecosystems such as the open ocean or oligotrophic lakes (Karl et al. 2008; Yao et al. 2016; Wang et al. 2017; Li et al. 2020). MPn is the simplest form of organic C-P bonded compounds in aquatic ecosystems (Karl et al. 2008); microbial utilization of Mpn, and the consequent breakdown of the C-P bond in this molecule, releases methane as a by-product (Kononova & Nesmeyanova 2002; White & Metcalf 2007). A number of marine and freshwater bacteria have the genomic potential to metabolize MPn and produce CH_4, based on the presence of the C-P lyase pathway. This includes Proteobacteria, Firmicutes, Bacteroidetes, Chloroflexi and Cyanobacteria (Kononova & Nesmeyanova 2002; Huang et al. 2005; Carini et al. 2014; Sosa et al. 2019), and expression of this pathway could potentially be regulated by phosphate availability (Kononova & Nesmeyanova 2002; Huang et al. 2005; White & Metcalf 2007; Sosa et al. 2019). Finally, recent work indicates that Cyanobacteria can directly produce methane during photosynthesis (Bižić et al. 2020). However, the exact mechanism by which this occurs remains unknown, and this has not been directly measured in aquatic ecosystems.

These proposed mechanisms for CH_4 production—(1) methanogenesis aided by detoxifying genes or in anoxic microsites, (2) methane production by breakdown of methylated compounds, and (3) methane production by Cyanobacteria—point to a diversity of ways CH_4 can be produced under oxygenated conditions. Many of these mechanisms are recently discovered and therefore poorly understood, and the degree

to which they occur within different aquatic ecosystems is largely unknown. In this study, we developed an experimental approach to disentangle these mechanisms and determine which may produce CH_4 in surface waters of freshwater lakes. We incubated freshwater from high-elevation lakes and measured CH_4 over time to assess net methane production or consumption. The use of incubations ruled out physical transport and allowed us to focus on potential biological mechanisms of oxic CH_4 production. We investigated specific mechanisms using a combination of biogeochemical measurements, experimental treatments and inhibitors, stable isotope analyses, and 16S rRNA, metagenome, and metatranscriptome sequencing. Paradoxical CH_4 production was evident in multiple experiments and experimental treatments, and could be attributed to MPn breakdown via widely-distributed members of the *Comamonadaceae* family. However, experimental treatments, stable isotope $\delta^{13}C$ signatures of CH_4, and metatranscriptomic data also point to a new potential mechanism of aerobic CH_4 production carried out by Cyanobacteria and Proteobacteria.

3.3 Materials and methods

3.3.1 Field site, sample collection, and experimental set up

Water samples were collected in 2016-2018 in five high-elevation lakes in Yosemite National Park, and used in incubation experiments. Samples were collected in Lukens (L), Lower Cathedral (LC), Upper Cathedral (UC), Lower Gaylor (LG), and Upper Gaylor (UG) Lakes; incubations are denoted by lake abbreviation and sequential numbering (Table 3.1). Water samples were collected in the littoral and limnetic zones of the lakes at 0.1 m depth with previously acid-washed plastic cubitainers, and then kept on ice or refrigeration to maintain in-lake temperature until laboratory incubations were established within 24 hours of samples collection. Temperature and dissolved oxygen were measured at the time of sample collection using a ProODO YSI probe (YSI Inc., Yellow Springs, OH, USA).

Water collected in the lakes was transferred to 170 or 300 ml Wheaton bottles, capped and crimped, and a known volume of air was introduced to generate a headspace for sampling. Initial CH_4 samples were collected, and bottles were incubated in water baths at constant temperature. All incubations were run in triplicate and set to the temperature at which water samples were collected in the field. The different treatments used in the experiments were:

- Control: unamended lake water following a natural day-night set up (water bath lid was opened at 7:00 hours and closed at 18:00 hours).
- Dark: unamended lake water; bottles were kept in the dark during the whole incubation time.
- BES: lake water was amended with 2-bromoethanesulphonate (BES) to a final concentration of $5x10^{-4}$ M. This concentration has been established to inhibit methanogens (Oremland & Capone 1988) and has been used in methane

paradox experimentation before (Grossart et al. 2011). This treatment followed the same natural daylight set up than the control.

- High light: unamended lake water was subjected to 500 mmol/m^2 inside a growth chamber and followed the same natural daylight set up than the control (light would turn on at 7:00 hours and off at 18:00 hours).

For all incubation types, gas samples were taken from the headspace every 6-24 hours for up to 96 hours with a syringe and immediately transferred to exetainers for later analyses in a gas chromatograph. Not all treatments were tested in each incubation experiment. Temperature and oxygen concentrations were monitored at each sampling point. Optical sensor spots (Fibox, Loligo Systems, Viborg, Denmark) were used to measure oxygen concentrations during incubations (detection limit of 100 nM) and make sure that the water did not go anoxic at any time. Temperature was measured with a Fibox temperature sensor and kept constant during the incubation time. Water samples were filtered at the beginning and end of the incubation for DNA and RNA sampling.

3.3.2 Methane measurements

Methane concentrations were measured via headspace equilibration and gas chromatography. Headspace gas samples from incubations were collected with a gas-tight syringe into 12-mL Labco Exetainer vials (Labco Ltd., Lampeter, Ceredigion, UK) after incubations bottles were shaken for 2 minutes to reach equilibration. Samples were later analyzed using a Shimadzu GC-2014 gas chromatograph with flame ionization detection (FID) for CH_4 (Weiss 1981). Headspace CH_4 concentration measurements were then used to calculate CH_4 concentration in lake water based on Henry's law of equilibrium (Yamamoto et al. 1976).

3.3.3 DNA and RNA extraction

Water samples were filtered through 0.22 μm (Millipore, Darmstadt, Germany) then DNA filter samples were preserved in Sucrose-Tris-EDTA (STE) buffer in pre-prepped Lysis Matrix E tubes and frozen at -80°C until extraction. RNA samples were preserved in RNA*later*® (Ambion™, AM7021) in pre-prepped Lysing Matrix E tubes (MP Bio, Eschwege, Germany), and frozen at -80°C until extraction. DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit with a modified protocol from Beman et al. (2012). Briefly, samples were lysed with 100μL 10% sodium dodecyl sulfate (SDS), DNA gets separated from proteins and cellular debris using proteinase K (20mg mL^{-1}; Qiagen, Inc., Valencia, CA, USA), precipitated with ethanol and cleaned up. After extraction samples were preserved at -80°C until further analyses. RNA was extracted using a *mir*Vana miRNA Isolation Kit (Ambion™, AM1560) with a modified protocol from Huber & Fortunato (2017). Briefly, samples are lysed with the kit's lysing matrix, then subjected to an organic extraction with phenol chloroform followed by a wash to obtain RNA. Immediately after RNA extraction we used the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies Corporation, Carlsbad, CA, USA) to synthesize first-

strand cDNA and samples were preserved at -80°C until further analyses. DNA, RNA and cDNA purity was measured using a Biospectrometer (Eppendorf AG, Hamburg, Germany) and the concentrations were quantified using PicoGreen Quant-iT dsDNA quantitation assay (ThermoFisher Scientific, USA) for DNA samples and the MaestroNano Pro (Maestrogen Inc., Taiwan) for RNA and cDNA samples.

3.3.4 16S sequencing

DNA and RNA extracted from filtered water samples were diluted to a common concentration (1ng/ul) and sent for 16S rRNA amplicon sequencing on an Illumina MiSeq (Illumina, San Diego, CA, USA) according to Earth Microbiome protocols. We used the universal primers 515F-Y (5'- GTGYCAGCMGCCGCGGTAA) and 926R (5'-CCGYCAATTYMTTTRAGTTT). DNA samples were sequenced at the Joint Genome Institute (Berkeley, CA, USA) and RNA samples at the Argonne National Laboratory (Lemont, IL, USA).

ASVs were generated from 16S rDNA and rRNA sequence data using the Divisive Amplicon Denoising Algorithm (DADA2; Callahan et al. 2016) as implemented in QIIME 2 (Bolyen et al. 2019), and then used for subsequent analyses. After import and demultiplexing, read quality was visualized using the 'qiime tools view' command. Reads were then processed using the 'qiime dada2 denoise-paired' command, with 13 bp trimmed from both the forward and reverse reads, truncation of reverse to 169 bp (due to the well-known decline in sequence quality observed for MiSeq reverse reads), and training of the denoising algorithm on 1 million reads. Classification of ASVs was conducted in mothur (Schloss et al. 2009) using the SILVA (version 128) database.

3.3.5 Metatranscriptomes and metagenomes

Metatranscriptomes and metagenomes were generated from later experiments in order to examine potential production mechanisms and coupled methane oxidation. Following extraction, DNA and RNA samples were sent for metagenome/metatranscriptome sequencing in the Vincent J. Coates Genome Sequencing Laboratory (GSL) at the University of California, Berkeley (https://genomics.qb3.berkeley.edu/), which is supported by NIH S10 OD018174 Instrumentation Grant. For each DNA sample, 250 ng of genomic DNA was sheared and libraries were prepared using the KAPA HyperPrep Kit (Kapa Biosystems, Wilmington, MA, USA). For each RNA sample, ~800 ng of total RNA was depleted of rRNA using the Ribo-Zero rRNA Removal Kit (Illumina, Inc., San Diego, CA, USA), sheared, and libraries were prepared using the KAPA RNA HyperPrep Kit (Kapa Biosystems, Wilmington, MA, USA). 12 samples were pooled into a single lane and sequenced via 150-cycle paired-end sequencing on the Illumina HiSeq 4000 platform (Illumina, Inc., San Diego, CA, USA).

Data were demultiplexed by the GSL and reads were filtered and trimmed using BBDuk (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/) with the following parameters: maq=8, maxns=1, minlen=40,minlenfraction=0.6,

k=23, hdist=1, trimq=12, qtrim=rl. Forward and reverse reads were then merged using PANDASeq (https://github.com/neufeld/pandaseq; Masella et al. 2012) with default parameters. Merged reads were subsequently annotated in DIAMOND (http://diamondsearch.org/; Buchfink et al. 2015) using the NCBI NR database (accessed February 11th, 2020) with the following search criteria: maximum number of target sequences = 1, bit-score > 40. In order to quantify functional gene abundances, we filtered the DIAMOND annotation data (Percent identity > 60%, bit score > 100) to find the number of functional genes of interest present in each metagenome or metatrascriptome. We calculated percent abundance based on the total number of reads and the number of targeted genes in each metagenome or metatranscriptome.

UC4 metatranscriptomes were uploaded to the Joint Genome Institute (JGI; https://img.jgi.doe.gov/) Integrated Microbial Genomes & Microbiomes platform for further analyses. We used the function comparisons tool to identify functions that were significantly different in the treatments vs. the control, and the function category tool identify differences in KEGG pathway categories among the treatments. Moreover, we used the phylogenetic distribution tool to quantify the abundance of different phylogenetic groups of potential importance in the methane paradox.

3.3.6 Stable isotopic measurements

Headspace of incubation bottles was transferred to evacuated exetainers and sent to the UC Davis Stable Isotope Facility (Davis, CA) for analysis. Measurements of stable isotope ratios of carbon ($\delta^{13}C$) in CH_4 were conducted using a ThermoScientific Precon concentration unit interfaced to a ThermoScientific Delta V Plus isotope ratio mass spectrometer (ThermoScientific, Bremen, Germany). In brief, gas samples are passed through a H_2O/ CO_2 scrubber and a cold trap, and CH_4 is then separated from other gases and oxidized to CO_2. Pure CO_2 reference gas is used to calculate provisional δ values, and final δ values are calculated after correcting for changes in linearity and instrumental drift, and expressed relative to the Vienna PeeDee Belemnite (V-PDB) standard.

3.3.7 Statistical analyses

Spearman's correlation test was used to assess the relationship between of time (h) and CH_4 concentration in all incubations and across all treatments. A priori significance level was defined as $\alpha<0.05$. A significantly positive relationship between time and CH_4 was considered as net CH_4 production in the incubation, whereas a significantly negative relationship was considered as net CH_4 consumption; otherwise we defined the incubation as not having significant net production or consumption over time. We subsequently used ANOVA and Tukey honestly significant difference (HSD) post-hoc tests to test for cases where CH_4 production or oxidation varied significantly between sampling time points, resulting in nonlinear patterns in CH_4 concentrations over the course of the experiment. For example, initial CH_4 production could be followed by subsequent oxidation, resulting in significant CH_4 increases followed by significant decreases.

To express the different responses in terms of CH_4 concentration over time among the different experiments treatments we first calculated the response ratios (lnRR) by the following equation:

$$lnRR = \ln \frac{mean\ CH_4\ in\ treatment}{mean\ CH_4\ in\ control}$$

Additionally, we calculated which treatments were significantly different from each other and the control using ANOVA and Tukey honestly significant difference (HSD) post-hoc tests.

All statistical analyses and figures were done in the R statistical environment (RStudioVersion 1.2.5001).

3.4 Results and discussion

Our experiments provide evidence that paradoxical methane production is widespread in freshwater lakes. Out of 19 total experiments conducted in five lakes in Yosemite National Park, 26% of experiments showed unequivocal, monotonic methane production, 16% showed net oxidation, and several showed significant nonlinear patterns (see below; based on replicate control bottles incubated for at least 24 hours; Fig. 3.1). We observed particularly high CH_4 production in Lukens Lake (e.g., L1 and L2 experiments), as well as consistent production in Lower Gaylor Lake (LG3 and LG4), and production within Upper Cathedral Lake (UC1). Net methane production rates ranged from 0.98 to 22.8 nM/h, with the majority of values <4 nM/h. These rates are consistent with the limited experiments previously conducted in other freshwater lakes (e.g., 0.1-2.5 nM/h and 3.7 in Lake Stechlin, Germany; Grossart et al. 2011 and Tang et al. 2014 respectively), and, on the higher end, our values are similar to the range reported by Bogard et al. (2014) for experimental manipulations in Lac Cromwell (2.08-8.33 nM/h). CH_4 turnover rates also ranged from 5 to 146 days (with in situ concentrations ranging from 309-2839 nM)—consistent with turnover rates of ~18 days in Lake Stechlin (CH_4 concentration ~430 nM, Grossart et al. 2011), ~2.2 days in Lac Cromwell (CH_4 concentration ~200 nM, Bogard et al. 2014), and 67 days in Yellowstone Lake (CH_4 concentration of 46.3 nM, Wang et al. 2017).

Our study is the first confirmation of the methane paradox in high elevation lakes, again demonstrating significant CH_4 production under oxygenated conditions. Incubations were monitored to confirm they were under oxic conditions at all times, and observed CH_4 production is likely biotic, as sterile treatments (filtered lake water) did not show significant CH_4 production or oxidation over time. Methane accumulation was consistently observed in multiple lakes, occurring across different experiments conducted in different years (2016, 2017 and 2018). However, our experimental results are also clearly indicative of natural spatial and temporal variation in CH_4 production and oxidation. For example, variations over time within

incubation experiments, as well as variation between and within experimental treatments, sometimes led to a lack of statistical significance—e.g., LG2 showed notable accumulation of methane which was not statistically significant due to variation across replicates.

However, variations within and across can provide insight into methane production mechanisms and their relation to methane consumption. Eleven experiments did not show monotonic increases or decreases, which could reflect either (1) a complete absence of CH_4 metabolism at the time of sampling, or, more likely, (2) variations in the balance between co-occurring CH_4 production and consumption over time. For example, initial CH_4 production could be followed by oxidation once CH_4 concentrations reach a certain threshold required for oxidation (Grossart et al. 2011). Conversely, initial decreases due to oxidation may be followed by eventual production—for example through the development of P limitation that triggers CH_4 production via MPn metabolism (Karl et al. 2008). It is also possible that production and oxidation proceed at similar rates, leading to no net change, even though CH_4 is actively cycled. In this case, molecular data are useful for providing insight into the underlying dynamics. We tested for nonlinear patterns using ANOVA, and found two cases of initial CH_4 production followed by oxidation (UC3 and LG1; Figure 3.2). We also examined CH_4 oxidation—manifested by the expression of the particulate methane monooxygenase gene *(pmoA)* in metatranscriptomes—and found that methane oxidation occurred in the majority of the incubations surveyed. These data, as well as observed variations in CH_4 concentrations, are indicative of active CH_4 oxidation that may obscure paradoxical production. Put another way, paradoxical CH_4 production is clearly taking place in incubations showing initial increases (UC3, LG1) or significant net increases (L1, L2, LG3, LG4 and UC1), but it could also occur in those experiments showing no significant change (or consumption) over time if CH_4 oxidation rates are equal to or greater than production rates. We therefore evaluated potential paradoxical CH_4 production mechanisms across experiments using multiple experimental treatments (BES, Dark, High-light), and applied 16S rRNA sequencing, metagenomics, metatranscriptomics, and $\delta^{13}C$ stable isotopic analyses to samples collected during these experiments.

3.4.1 Methanogenesis

We used these multiple approaches to test for phytoplankton- or particle-based methanogenesis, as initial observations in freshwater lakes showed potential acetoclastic methanogens attached to phytoplankton (Grossart et al. 2011), while other work has suggested methane production can occur in anoxic microsites on particles (Oremland 1979; Traganza et al. 1979; Oremland & Polcin 1982; King et al. 1983; Angelis & Lee 1994; Karl & Tilbrook 1994). In all experiments from 2017 and 2018, we included a treatment that consisted of the addition of the methanogenesis inhibitor BES (2-bromoethanesulphonate). While there are some caveats with its use (Oremland & Capone 1988), BES is widely used, including earlier tests of the methane paradox (Grossart et al. 2011; Tang et al. 2014). In our experiments, BES had an inhibitory effect on CH_4 production in four experiments, but increased CH_4

production rates in four experiments, and had no significant effect in the 5 remaining experiments. 16S rRNA sequencing of DNA from experiments L1-6 and LG1-4 recovered no methanogen 16S sequences (out of 5.4 million total sequences). Sequencing of 16S rRNA transcripts in RNA samples from experiments L5-6, LG5-6, and UC3-4 recovered methanogen sequences in only two samples (dark treatments from L5 and LG5), but total numbers were only 0.014% and 0.008% of all 16S rRNA sequences. Analysis of metatranscriptomes likewise showed that methyl coenzyme A (*mcrA*; responsible for methanogenesis) transcripts were absent in LG6 and UC4(tf) incubations and minimal in LG5, L7 and UC4(t0) (Fig. 3.3). *mcrA* genes were absent in all metagenomes, with the exception of the L6(tf) incubation (Fig. 3.3). Collectively these data indicate that water column-based methanogenesis is not a substantial source of CH_4, as BES effects were mixed, methanogens were almost entirely absent and inactive based on 16S data, and *mcrA* transcripts and genes were scantly expressed in metatranscriptomes and rarely detected in metagenomes.

3.4.2 Methylphosphonate breakdown as a source of methane

Analysis of metatranscriptomes and metagenomes showed potential for CH_4 production mechanisms other than conventional methanogenesis. In particular, we found evidence for microbial metabolism of MPn leading to CH_4 formation based on the universal expression of alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase (*phnJ*) transcripts in metatranscriptomes (Fig. 3.3). *phnJ* is responsible for the cleavage of the C-P bond that results in CH_4 production from MPn; this mechanism is thought to be the dominant paradoxical CH_4 production mechanism in the ocean (Repeta et al. 2016), but has been documented in only a limited number of freshwater lakes (Yao et al. 2016; Wang et al. 2017; Li et al. 2020). In addition to *phnJ*, phosphonates-binding periplasmic protein (*phnD)* genes, (involved in the binding component of phosphonate uptake; Rizk et al. 2006) were also universally expressed (Fig. 3.3). *phnJ* and *phnD* genes were also recovered in all metagenomes (Fig. 3.3), and the majority of the genes were present in organisms of the same genera as the metatranscriptomes. Widespread expression of *phnJ* was observed predominantly in organisms of the genus *Mucilaginibacter* (77 - 95% identity), *Polaromonas* (87-98% identity), *Variovorax* (80-94% identity), *Methylovirgula* (76.5-95% identity), and particularly *Limnohabitans* (95.5-100 % identity)—suggesting that phosphorus assimilation from MPn is broadly distributed among surface water microbes in these lakes.

Related to this, we examined whether transcripts and genes involved in the production of MPn were also present and active. Transcripts of phosphoenolpyruvate mutase (*pepM*) and phosphonopyruvate decarboxylase (*ppd*), two major genes involved in phosphonate biosynthesis (Yu et al. 2013), were present in some of the metatranscriptomes (Fig. 3.3), which suggests that synthesis of phosphonate groups also occurs within these lakes. Moreover and unexpectedly, genes involved in the DMSP demethylation pathway (*dmdB*: 3-methylmercaptopropionyl-CoA ligase, *dmdC*: 3-methylmercaptopropionyl-CoA dehydrogenase and, *dmdD*: Methylthioacryloyl-CoA hydratase) were expressed in some of the

metatranscriptomes. The role of DMSP degradation pathways in the methane paradox in the ocean has already been hypothesized—for example, the *dmdD* gene involved in the production of methanethiol (MeSH) has been proposed to be a precursor for methane paradox in polar waters subject to nutrient availability (Kiene et al. 2000, Damm et al. 2010, Moran et al. 2012). *dmdD* was expressed in only in one of our incubations (LG5) and not detected in any metagenomes, whereas *dmdC* was more commonly expressed (exceptions LG5 and UC4-HL) and present in all metagenomes (Fig. 3.3). Dimethylsulfoniopropionate demethylase (*dmdA*; involved in the first step in DMSP breakdown) and *dmdB* genes were also present in L6 and LG2 incubations (Fig. 3.3). Few studies have examined DMSP degradation in lakes, although DMS concentrations in freshwater can be similar to those found in oceans (Sela-Adler et al. 2015). Our findings suggest that members of the Actinobacteria phylum and *Comamonadaceae* family may be involved in DMSP transformations in lakes based on metatranscriptomic data, and at least raise the possibility of DMS and CH_4 production from DMSP.

The majority of the *phnJ* transcripts and genes were also found among organisms in the *Comamonadaceae* family, and 16S data demonstrate that *Comamonadaceae* were consistently present and abundant across experiments. Since the genes involved in phosphonate acquisition and utilization often occur in clusters, we assembled and annotated metagenomes and metatranscriptomes to examine whether particular groups possess and express multiple *phn* genes in parallel. *Comamonadaceae* were common in all metatranscriptomes and metagenomes and we found several *phn* gene clusters were affiliated to this family. For example, contigs containing genes *phnC*, *phnD*, *phnE*, *phnI*, *phnJ*, *phnK*, *phnL*, and *phnM*, were present in metagenomes from incubations LG2 and L6 in organisms affiliated to the abundant freshwater bacterial group *Limnohabitans,* as well as other members of the *Comamonadaceae* such as *Hydrogenophaga* and *Acidovorax.*

Phosphorus limitation is common in freshwater lakes, as it is in the open ocean (Björkman & Karl 2003; Sickman et al. 2003; Elser et al. 2009). High-elevation lakes in the Sierra Nevada are typically oligotrophic (Sickman et al. 2003; Moser et al. 2019), and inorganic phosphate concentrations in the lakes sampled here are consistently near the limits of detection (100 nM) by colorimetric techniques—with 79% of measurements <200 nM (Hayden & Beman 2016; Chapter 2). Under these conditions, the use of organic P sources, including phosphonates, could be widespread. Our findings are consistent with this idea, and the widespread presence and expression of *phnJ* genes indicates that several microbial groups are capable of releasing CH_4 through the cleavage of the C-P bond in MPn.

3.4.3 Evidence for methane production by Cyanobacteria and Proteobacteria

In addition to microbial use of MPn, Cyanobacteria were recently identified as a possible source of paradoxical CH_4 production—yet this has not been specifically investigated in the environment, and the pathway by which Cyanobacteria ultimately produce CH_4 remains unknown (Bižić et al. 2020). Based on 16S rRNA genes and

transcripts, and metagenomes and metatranscriptomes, Cyanobacteria were always detectable in our samples. However, Cyanobacteria were also variable across lakes, over time in individual experiments, and especially between experimental treatments. We evaluated whether these variations could be used to determine whether Cyanobacteria are a significant source of paradoxical CH_4 production in freshwater.

In particular, two additional treatments were used to partition the relative effects of CH_4 production vs. oxidation and evaluate Cyanobacteria as a potential source of CH_4. First, we conducted light versus dark treatments in some experiments, as light has been shown to inhibit methane oxidation (Dumestre et al. 1999; Murase & Sugimoto 2005; Tang et al. 2014; Thottathil et al. 2018) and CH_4 production in cyanobacterial cultures was positively correlated with light (Bižić et al. 2020). Dark treatments would therefore be expected to have higher methane oxidation rates, lower cyanobacterial CH_4 production, and therefore lower CH_4 concentrations compared with controls. Additionally, we increased light intensity in four experiments, which may have a two-fold effect: (1) greater inhibition of methane oxidation, and (2) increased rates of CH_4 production from cyanobacteria or phytoplankton (Grossart et al. 2011; Bogard et al. 2014; Bižić et al. 2020). For both of these reasons, we expected to observe higher CH_4 production rates under higher light levels.

Dark bottles showed mixed results, with four cases of CH_4 consumption (L4, LC1and LG6 at 24 hours and LG5 at 86 hours; all $p<0.05$), production in two cases (UC2 at 24 hours and L5 at 74 hours; both $p<0.05$), and no change in four cases (L7 and LG4 at 24 hours, L3 at 36 hours and UC3 at 57 hours; all $p>0.05$). Where CH_4 concentrations decreased significantly in dark bottles compared with controls (Fig. 3.4), we estimated that oxidation rates increased 8 fold for L4 (24 h; $p<0.05$), whereas production rates decreased 3 fold in LG4 (24 h; $p<0.05$) and 22 fold in LG5 (72 h; $p<0.0005$). In two of four experiments with high light treatments, we observed increased CH_4 production (L2 and UC4; both $p<0.05$). The UC4 experiment was particularly illuminating, as higher light intensity significantly boosted CH_4 production rates by 62-fold compared with the control (up to 3.2 nM/h; $p<0.0005$). This was among the strongest treatment effects observed across all experiments (Fig. 3.4). BES also increased CH_4 production rate by 49-fold compared with control in this experiment (up to 2.5 nM/h; $p<0.0005$, Fig. 3.4).

To examine this in greater detail, we analyzed both the stable isotope composition of CH_4 produced during the UC4 experiment, as well as changes in gene expression in response to experimental treatments in metatranscriptomes. The stable isotope composition of CH_4 ($\delta^{13}C$) from multiple sampling time points in the BES and HL treatments was compared with controls in the UC4 experiment; $\delta^{13}C$ values in the LG6 and L7 experiments (which did not show CH_4 production in treatments) were also compared (Fig. 3.5).We found that initial $\delta^{13}C$ values in UC4 (-50.86 ‰) and L7 (-51.88 ‰; Fig. 3.5) differed from atmospheric values (-45 to -47 ‰), consistent with an initial biological source of CH_4. LG6 values were heavier, which may reflect methane oxidation acting preferentially on the lighter isotope, enriching the

remaining CH_4 (methane oxidation imparts a fractionation effect of ~10 to 20‰; Venkiteswaran & Schiff 2005). L7 and LG6 experiments showed no significant variation in $\delta^{13}C$ between treatments, in line with little change in CH_4 concentrations.

UC4 data presented a clear contrast, as $\delta^{13}C$ values were significantly lower in the BES and HL treatments compared with controls. Along with increased CH_4 concentrations, these data are indicative of production of isotopically-depleted CH_4 in BES and HL treatments, which we calculate to be -56.9 ‰ and -57 ‰ respectively based on isotopic mass balance. Traditionally this could be interpreted as methanogenesis, as the $\delta^{13}C$ of biogenic CH_4 (-110 to -50 ‰) is lower than terrestrial organic material (-28 to -26 ‰ for C3 plants) or lake primary production (-35 to -25 ‰; Grey 2016). While methanogenesis cannot be entirely ruled out, low levels of *mcrA* expression (Fig. 3.3) in these treatments suggests another source. Strong isotopic fractionation from MPn also seems unlikely, as the mean isotopic fractionation for methane derived from MPn by the cleavage of the C-P bond is only 1.3‰ (Taenzer et al 2020). MPn could be allochthonous (terrestrial) or autochthonous (based on *pepM* results), but in either case, is unlikely to be so ^{13}C-depleted. For example, in marine systems, CH_4 production from MPn actually increases $\delta^{13}C$ (Repeta et al. 2016).

Instead, our isotopic data and metatranscriptomic data are consistent with Cyanobacteria as a source of CH_4 in the UC4 experiment. In cyanobacterial cultures, produced CH_4 was isotopically-depleted, with values (-45 to -55‰; Bižić et al., 2020) similar to those we observed. Only the addition of ^{13}C-labeled dissolved inorganic carbon (DIC) to cyanobacterial cultures resulted in higher $\delta^{13}C$-CH_4 values, demonstrating that CH_4 was ultimately derived from DIC. Although the pathway by which Cyanobacteria produce CH_4 remains unidentified, Bižić et al., (2020) hypothesized that CH_4 is produced during the photosynthetic process owing to positive correlation between light and CH_4. However, we found no significant differences in carbon fixation ($p>0.05$) or photosynthesis ($p>0.05$) KEGG Pathway categories between treatments in the UC4 experiment.

We consequently used metatranscriptomic data to identify specific functions that were significantly different in the treatments vs. the control, and that may explain differences in CH_4 concentrations and isotopic composition. This is an advantage of 'omic data, as it is possible to examine previously unidentified potential mechanisms for CH_4 production. Through comparison of all functions across metatranscriptomes from the UC4 experiment, we identified two functions, both involved in porphyrin and chlorophyll metabolism, that were upregulated: ferredoxin:protochlorophyllide reductase (DPOR) and chlorophyllide a reductase (COR) were significantly different between the control and the BES ($p<0.0005$) and HL ($p<0.005$) treatments (Fig.3.5). While not directly involved in the photosynthesis pathway, DPOR is found in photosynthetic bacteria, Cyanobacteria, and green algae, and is involved in the light independent reduction of protochlorophyllide (Fujita et al., 1993; Nomata et al., 2008); COR catalyzes the first step in the conversion of chlorin to a bacteriochlorin ring during bacteriochlorophyll biosynthesis (Nomata et al., 2006; Chew & Bryant,

2007; Tsukatani et al., 2013). Notably, both DPOR and COR are nitrogenase-like enzymes (Nomata et al., 2006; Chew & Bryant, 2007; Tsukatani et al., 2013), and both were abundant in all metatranscriptomes and metagenomes. DPOR and COR were particularly common within the *Limnohabitans* and *Polynucleobacter* genera, as some strains of these abundant freshwater bacteria are capable of performing aerobic anoxygenic photosynthesis (Kasalický et al., 2018; Imhoff et al., 2019; Hahn et al., 2017). DPOR exclusively was also found in several cyanobacterial groups—such as *Pseudanabaena*, *Dolichospermum*, *Gloeomargarita* and *Oscillatoria*—in the metatranscriptome data.

Based on these findings, we propose two potential mechanisms that could be involved in paradoxical CH_4 production in oxic surface waters of freshwater lakes: (1) methane may be produced from methoxyl groups present in (bacterio)chlorophyll precursors, or (2) methane production may be catalyzed by DPOR and COR enzymes. In terrestrial plants, CH_4 is thought to be produced aerobically from structural components—such as pectin, lignin and cellulose (Keppler et al., 2006; Messenger et al., 2009; Vigano et al., 2008)—mainly in plants under stress (such as increased temperature, UV radiation or physical damage; Bruhn et al., 2012). Protochlorophyllide, chlorophyllide, and other bacteriochlorophyll precursors contain methoxyl groups that have been shown to serve as precursors of CH_4 in plants (Keppler et al., 2008), and a similar mechanism may be active in Cyanobacteria and/or Proteobacteria under stress in our experimental treatments.

Alternatively, CH_4 production may be catalyzed by DPOR and COR enzymes. Nitrogenases reduce a range of multibond compounds (Hu et al., 2011; Yang et al., 2011; Lee et al., 2010; Zheng et al., 2018), and this quality is shared across different nitrogenases (Seefeldt et al., 2020). Although DPOR and COR were expressed and present in all metatranscriptomes and metagenomes, expression of both was an order of magnitude higher in the UC4 experiment compared with the LG5, LG6, and L7 experiments (with the exception of COR in LG5). This suggests that this mechanism can be variable, but is highly consistent with the lack of a $\delta^{13}CH_4$ isotopic signal in the LG6 and L7 experiments compared with the UC4 experiment (Fig. 3.4). We propose that, similar to the findings of Zheng et al. (2018), the nitrogenase-like enzymes DPOR and COR may be able to catalyze the reduction of CO_2 into CH_4. Importantly, these enzymes are present in Cyanobacteria as well as the abundant and ubiquitous freshwater bacterial groups *Limnohabitans* and *Polynucleobacter* (Kasalický et al., 2018; Imhoff et al., 2019; Hahn et al., 2017).

3.4.5 Multiple mechanisms of methane production

Altogether, our results indicate that multiple paradoxical CH_4 production mechanisms can occur in freshwater ecosystems, and have several implications for our understanding of aquatic CH_4 cycling. First, confirmation of the CH_4 paradox in freshwater is still limited in scope and relatively recent (Grossart et al. 2011; Bogard et al. 2014; Bižić et al. 2020). We conducted multiple experiments in multiple lakes,

and measured significant CH_4 production in 37% of experiments (Fig. 3.1). In many of the remaining experiments, variations over time, across experimental treatments, and in *pmoA* gene expression patterns suggest that CH_4 production likely occurs in parallel with coupled CH_4 oxidation (Fig. 3.2; Fig 3.3). Because CH_4 oxidation may obscure CH_4 production, use of different experimental treatments combined with 'omic data is required to identify paradoxical CH_4 production. Our findings therefore expand on limited data and demonstrate that paradoxical CH_4 production is common, but variable, in freshwater.

Given the ubiquity of *phnJ* genes in our data (Fig. 3.3), their presence on contigs from abundant organisms, and the potential for P limitation in these lakes, we suggest that microbial metabolism of MPn represents an important baseline CH_4 production mechanism in these freshwater lakes. This fits with current understanding of marine ecosystems (Karl et al. 2008; Repeta et al. 2016). Metatranscriptomic and metagenomic data also indicate that, like the ocean, DMSP transformations may be active in freshwater—with potential implications for both DMS and CH_4 production. Our data further demonstrate that MPn may be produced within lakes based on *ppd* and *pepM* expression. Quantifying the relative contributions of allochthonous vs. autochthonous MPn production—as well as broader P acquisition dynamics—is therefore essential to understanding CH_4 production in freshwater. Phosphonate compounds were once thought to be resistant to breakdown and their formation remains poorly understood (Kafarski, 2019). Under P limiting conditions, competition for these and other P compounds among multiple groups of organisms will be intense, and potentially variable in space and time. Along with variations in CH_4 oxidation relative to production rates, this will drive variations in CH_4 production such as those evident in our data.

As a newly-identified CH_4 source that has not been examined in the environment and occurs via an unknown pathway (Bižić et al. 2020), we used a combination of approaches to identify whether cyanobacterial CH_4 production occurs in freshwater, and by what means. Although independent data types (CH_4 concentrations, treatment effects, isotopic data, 'omic data) may be interpreted in multiple ways, the combined data are consistent with cyanobacterial production in the UC4 experiment. For example, the high light treatment (and BES) boosted CH_4 concentrations (Fig. 3.4), but this CH_4 is unlikely to have been the result of methanogenesis or MPn breakdown given low and decreasing *mcrA* and *phnJ* expression (Fig. 3.6). If methanogenesis is a source of CH_4 in these lakes, per transcript/gene rates would have to be remarkably high given 16S rRNA and *mcrA* numbers. Instead, Cyanobacteria were present and responsive to treatments as shown by metagenomic, metatranscriptomic, and 16S data. Our data are further indicative of an isotopically light C source (Fig. 3.5) consistent with $\delta^{13}C$ observations by Bižić et al. (2020).

Notably, these patterns were most clearly evident in experimental treatments, highlighting the efficacy of including different treatments to identify production mechanisms. We leveraged 'omic data from these different treatments to identify potential mechanisms of CH_4 production—providing an experimental approach,

additional isotopic signature data, and two potential gene targets to examine in other aquatic ecosystems. We found that this mechanism was not limited to the Cyanobacteria, as the majority of DPOR and COR transcripts and genes were derived from several members of the Proteobacteria. This has multiple implications, as *Limnohabitans* and *Polynucleobacter* are among the most abundant and ubiquitous bacteria found in freshwater ecosystems (Newton et al., 2011; Kasalický et al., 2018; Hahn et al., 2017; Imhoff et al., 2019). Although there is significant genomic diversity within these groups (Newton et al., 2011; Kasalický et al., 2018; Hahn et al., 2017), this suggests that the potential for CH_4 production via this pathway may be widespread in freshwater. *Limnohabitans* also expressed *phnJ* genes, indicating that they can play a dual role in paradoxical CH_4 production.

Ultimately, our combined dataset indicates that paradoxical CH_4 production is complex, with multiple interacting groups and processes, each affected by multiple environmental variables (e.g., P availability, light), and all producing CH_4 to differing degrees. Understanding this complexity is essential, as multiple paradoxical CH_4 production mechanisms are widely-distributed, and so may be important sources of CH_4 to the atmosphere.

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3.6 Tables

Table 3.1 Experimental incubations characteristics. BES corresponds to the addition of 2-bromoethanesulphonate as a methanogenesis inhibitor, Dark represents experiments incubated in total darkness and HL (high-light) are experiments subjected to 500 mmol/m^2 light intensity during the incubation.

Experiment	Y	M	D	CH_4	*P-value*	16S rDNA	16S rRNA	Metat	Metag	Length of incubation	Treatment
L1	2016	8	12-13	+	0.02	X				24	
L2	2016	9	11-12	+	0.02	X				24	HL
L3	2017	7	28-30			X				48	BES, Dark
L4	2017	8	8-10			X				48	BES, Dark
L5	2017	9	26-29	-	0.02	X	X			74	BES, Dark
L6	2017	11	13-15			X	X	X	X	55	BES
L7	2018	9	23-28	-	0.02		X	X		96	BES, Dark, HL
LG1	2016	8	8-9	+/-	0.02	X				24	
LG2	2016	9	9-10			X			X	24	
LG3	2016	10	7-8	+	0.02	X				24	
LG4	2017	8	24-26	+	0.02	X				48	BES, Dark
LG5	2017	10	1-4				X	X		86	BES, Dark
LG6	2018	9	10-14				X	X		96	BES, Dark, HL
UC1	2016	8	10-11	+	0.02					24	
UC2	2017	8	10-12							48	BES, Dark
UC3	2017	10	8-11	+/-	0.006		X			57	BES, Dark
UC4	2018	10	8-12				X	X		96	BES, Dark, HL
LC1	2017	08	26-28	-	0.02					48	BES, Dark
UG1	2017	08	12-14							48	BES

3.7 Figures

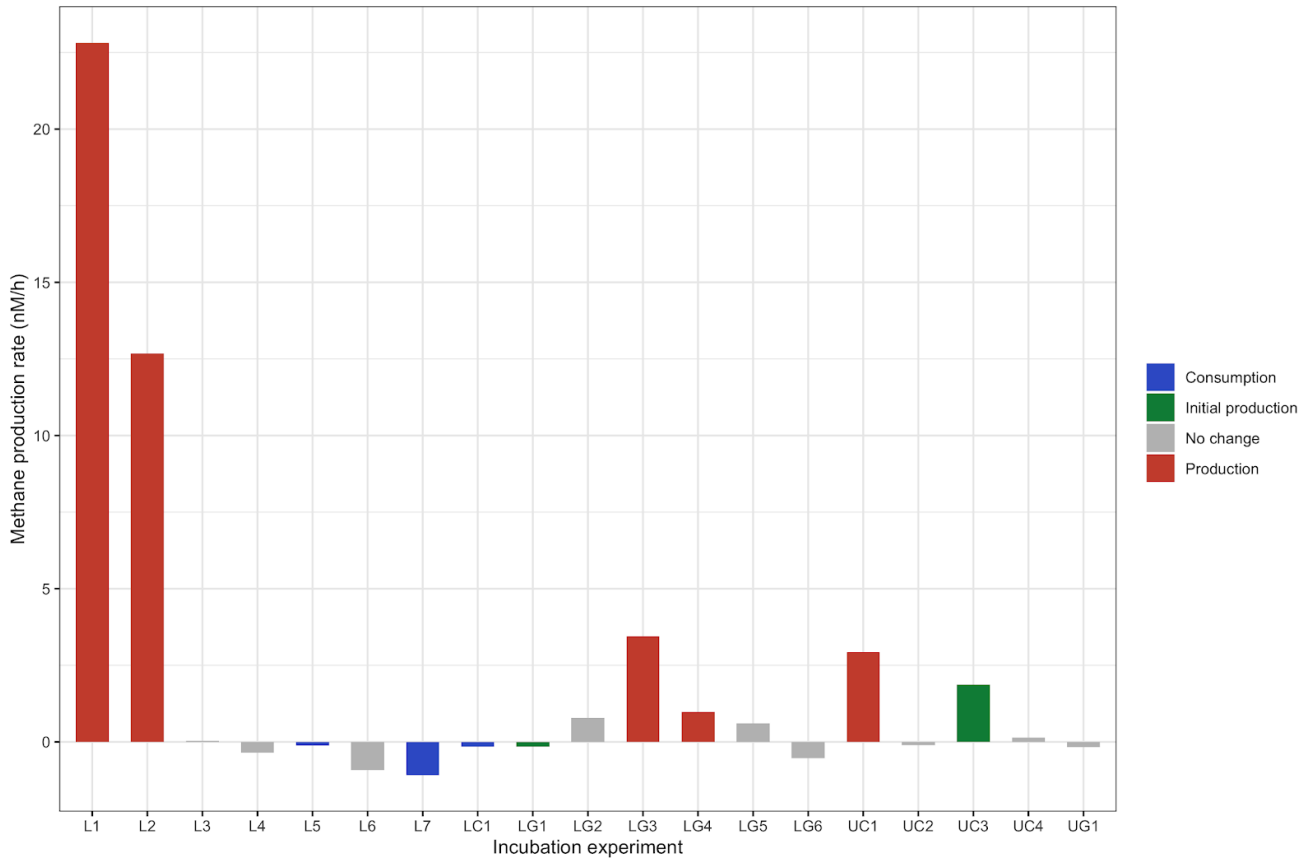

Figure 3.1 Net methane (CH_4) production or consumption rates across incubation experiments. Different colors represent significant linear increases (production; red), linear decreases (consumption; blue), nonlinear patterns (green), or no significant change in CH_4 concentrations (grey) over 24 hours in unamended controls.

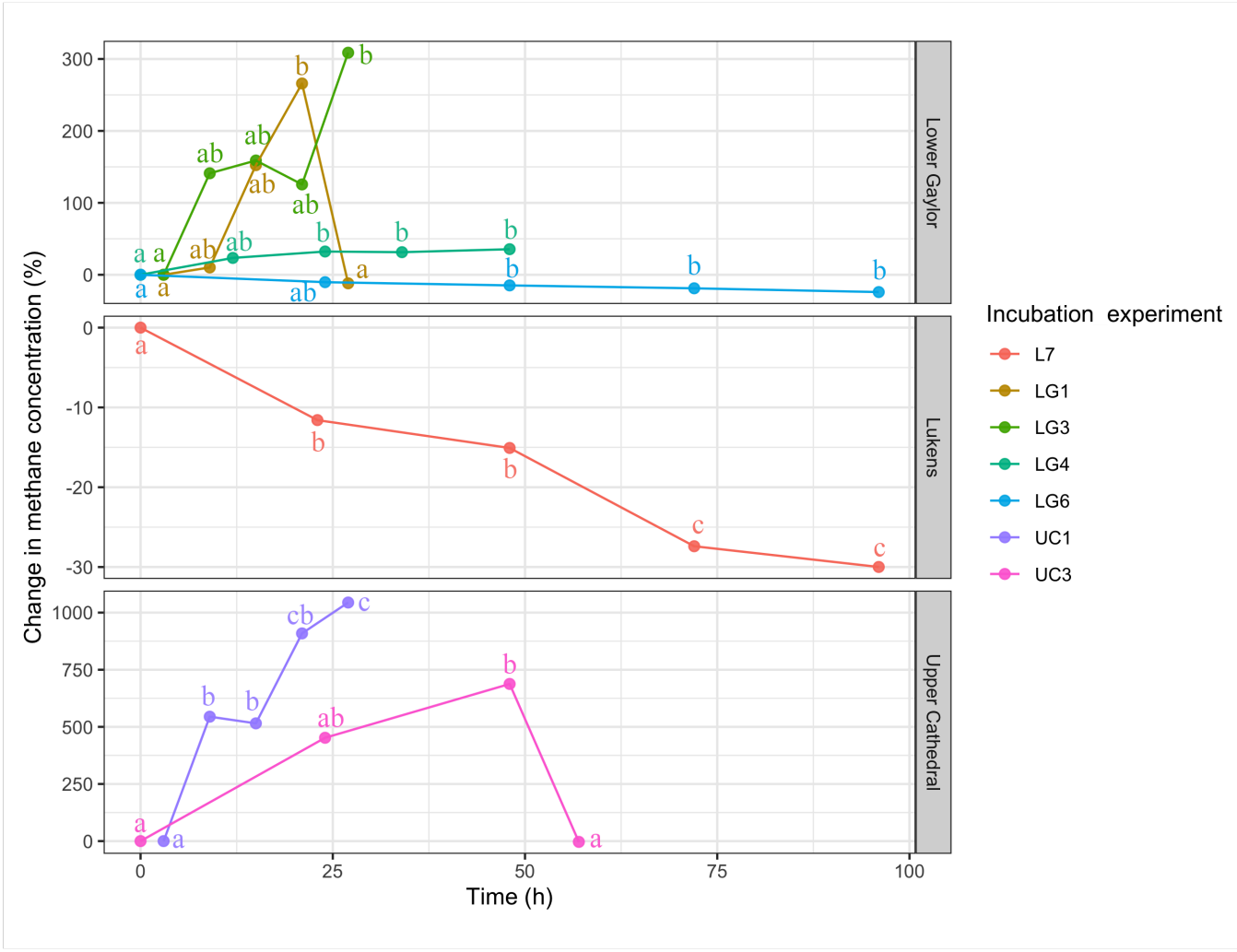

Figure 3.2 Percent change in methane concentrations over time. Different colors represent each incubation experiment with statistically significant differences in methane concentrations among different time points. Letters represent similarities or differences between time points according to Tukey HSD test.

Figure 3.3 Variations across experiments and treatments in (a) transcript abundance (% of total reads of metatranscriptomes) and (b) Gene abundance (% of total reads of metagenomes). Functional genes quantified involved in methane oxidation (*pmoA*), methanogenesis (*mcrA*), phosphonate assimilation (*phnD*, *phnJ*) and production (*ppd*, *pepM*), and DMSP metabolism (*dmdA*, *dmdB*, *dmdC*, *dmdD*).

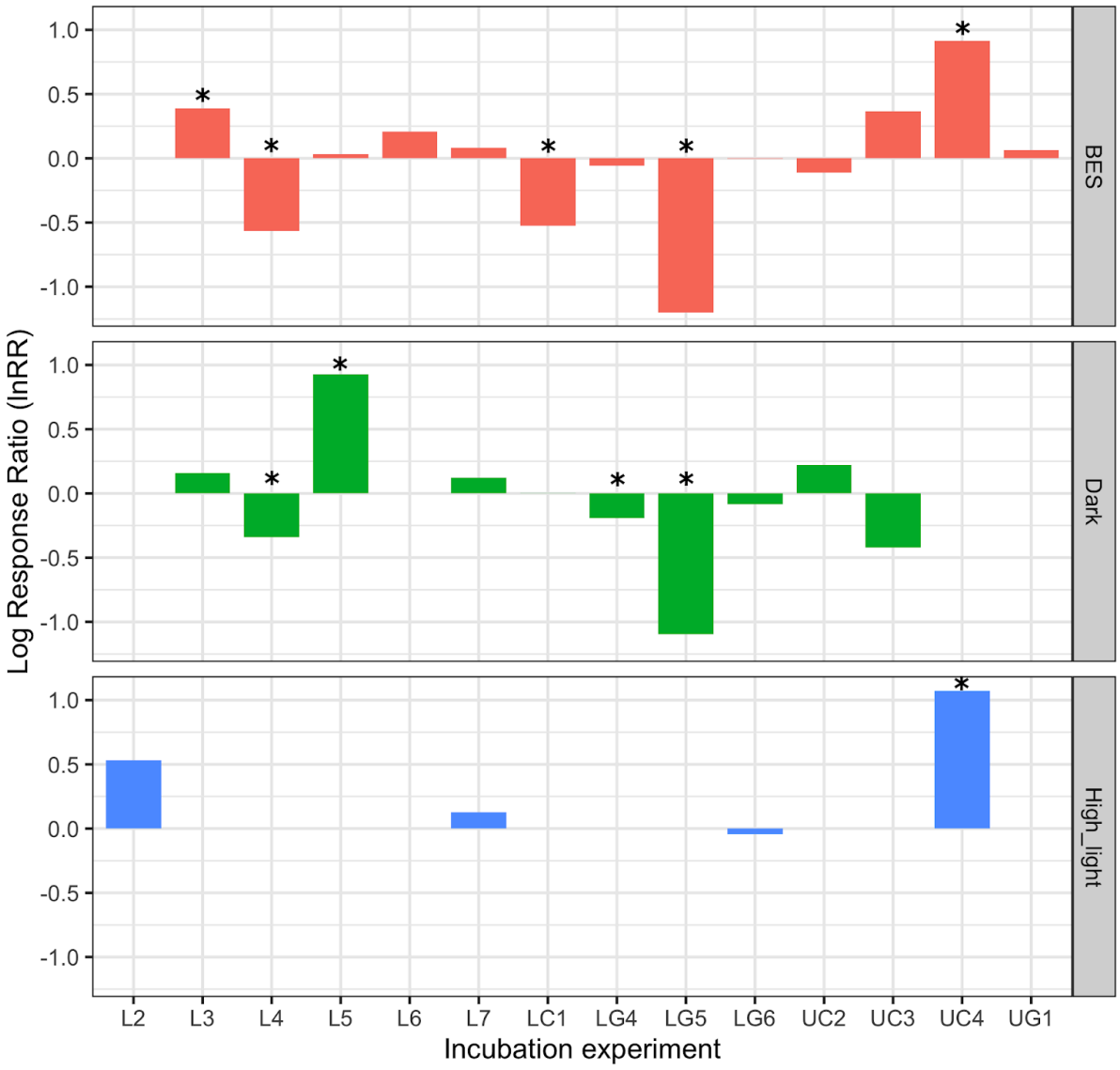

Figure 3.4 Log response ratios for experimental treatments at 24 hours. Asterisks denote experimental treatments that are significantly different from the control. Experiments L3, L5, LG5 and UC3 were longer experiments and treatment effects are shown for 36, 74, 86 and 57 hours respectively).

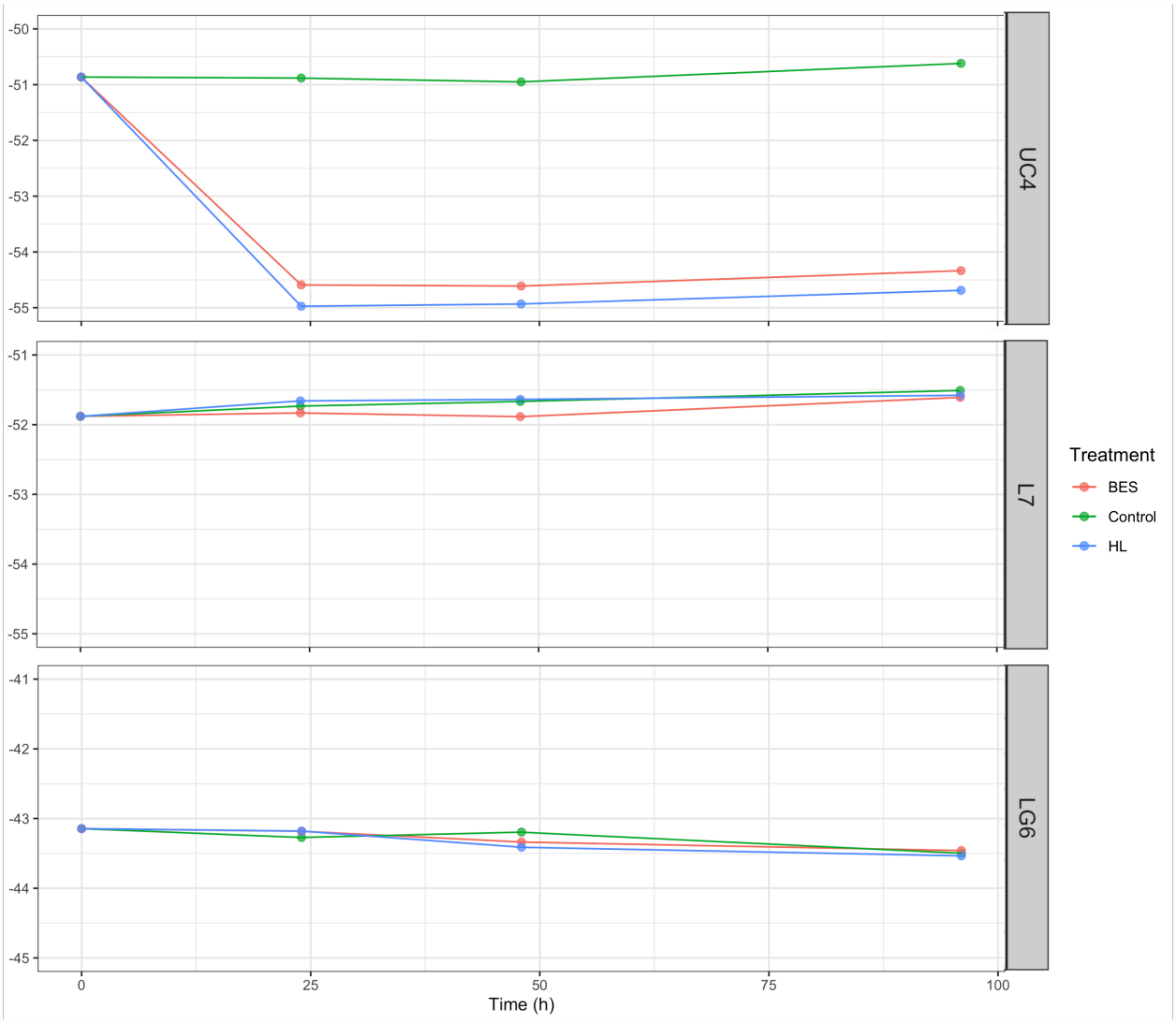

Figure 3.5 $\delta^{13}C$ values of dissolved methane over time in incubations L7, LG6 and UC4. Different colors denote different experimental treatments. Scales of the vertical axes differ between experiments.

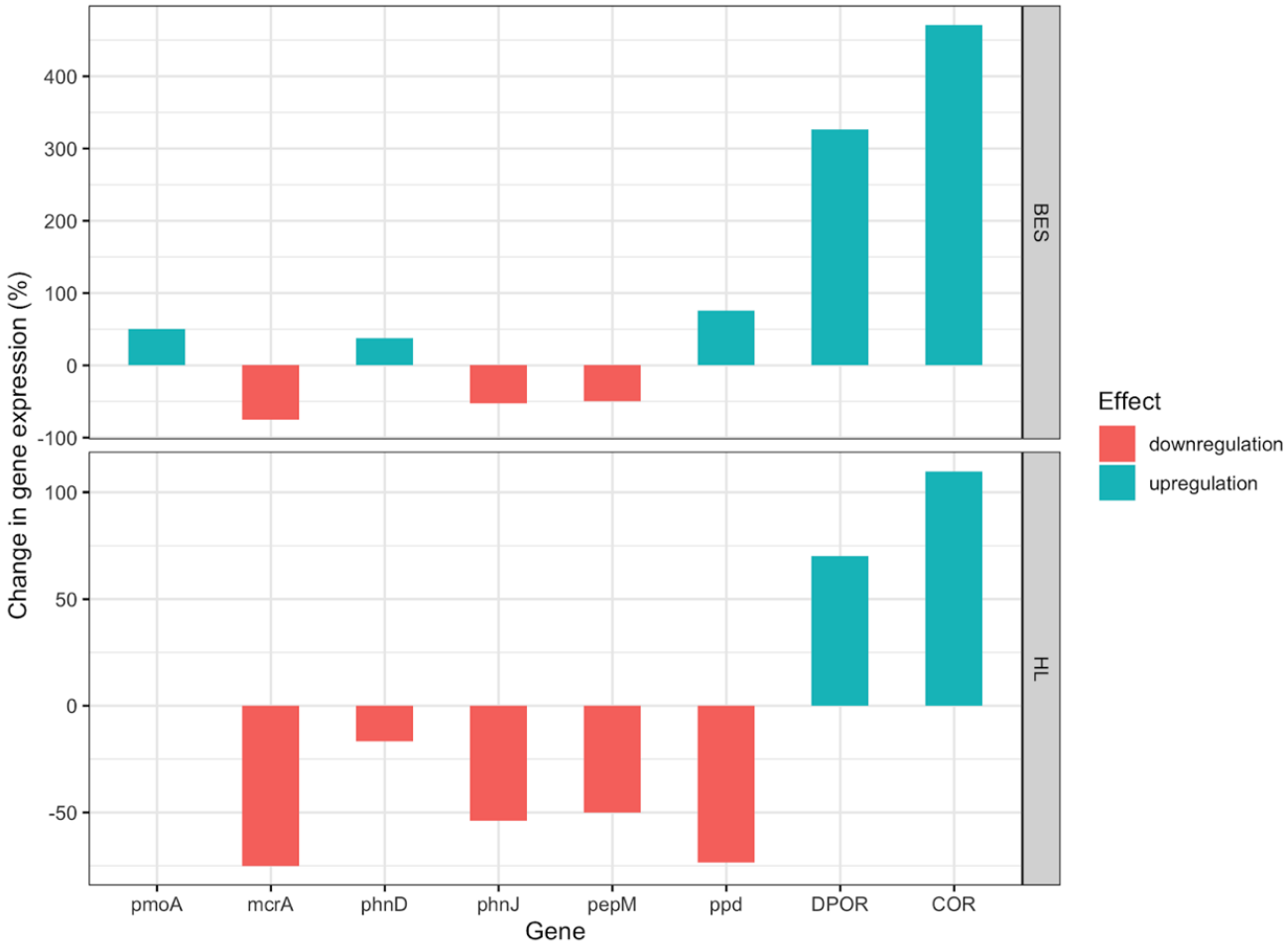

Figure 3.6 Up and downregulation of genes involved in methane oxidation (*pmoA*), methanogenesis *(mcrA*), phosphonate assimilation (*phnD*, *phnJ*) and synthesis (*pepM*, *ppd*), and porphyrin and chlorophyll metabolism (ferredoxin:protochlorophyllide reductase: DPOR and chlorophyllide a reductase: COR). BES treatment is shown in the top panel and the high-light treatment in the bottom panel.

4 Experimental warming can increase aerobic methane production and oxidation in surface waters of freshwater lakes

4.1 Abstract

Global temperatures are increasing due to anthropogenic induced climate change. Among the multiple biological processes impacted by warming, increased methane (CH_4) cycling rates could result in future positive climate feedbacks if increased temperature accelerates CH_4 production over CH_4 oxidation. As a result, it is critical to understand how microorganisms involved in the CH_4 cycle will respond to warming—and especially those that are currently poorly characterized, such as the microbes involved in 'paradoxical' CH_4 production. In this study we incubated lake water samples under both natural and increased temperatures to address the effect of warming on water column CH_4 cycling. Our findings suggest that aerobic CH_4 production can be positively affected by warming, as shown by the upregulation of *phnJ*, DPOR, and COR genes, as well as significant CH_4 increases in our experiments. CH_4 oxidation was also active and especially *mmo* expression was positively responsive to warming, and we observed significant CH_4 consumption in some experiments. This study shows for the first time that paradoxical CH_4 production mechanisms are sensitive to warming, and suggests that these largely unaccounted sources could increase as global temperatures rise.

4.2 Introduction

Methane (CH_4) is the second-most important greenhouse gas in Earth's atmosphere, with a global warming potential 28 times that of carbon dioxide (IPCC 2014). Methane therefore holds an important role in the radiative forcing of the planet, and understanding the CH_4 component of the carbon cycle is important for predicting further alterations in global climate (Saunois et al. 2016). Atmospheric methane concentrations have also increased significantly since pre-industrial times due to human alteration of the global carbon cycle (Conrad 2009). However, an important but uncertain aspect of CH_4 biogeochemistry is the potential for positive feedbacks that result in increased CH_4 flux in response to climate change. Multiple biological processes produce and consume CH_4 in different ecosystems, and the temperature sensitivity of these processes is poorly constrained.

Of all ecosystems, freshwater lakes represent a particularly important natural source of CH_4, with their contribution to the methane budget estimated to be as high as 6-16% of natural CH_4 emissions, despite only covering 0.9% of the Earth's surface (Bastviken et al., 2004). Methane flux to the atmosphere from lakes is conventionally thought to reflect the balance between two microbial processes: methanogenesis—the anaerobic process that produces CH_4 in anoxic sediments—and methane oxidation—which consumes 30-99% of produced CH_4 at the sediment-water interface and in the water column (Bastviken et al., 2004). Methane flux to the atmosphere represents the difference between what is produced and consumed.

However, a growing number of studies have documented CH_4 production in the oxic water column as a widespread process in freshwater (Bogard et al., 2014) and marine ecosystems (Karl et al., 2008). This phenomenon is often referred to as the "methane paradox", and while the underlying mechanisms of how this happens are not completely clear, it appears to be a significant contributor to the total lake methane efflux (Tang et al., 2016). The methane paradox has major implications for the CH_4 budget because CH_4 produced in the water column is less likely to be oxidized (due to the potential light inhibition of methane-oxidation; Murase & Sugimoto, 2005), and can reach the surface and be emitted more readily (Grossart et al., 2011).

A major open question is how the balance between CH_4 production and consumption may change as a function of changing temperature—especially in climate-sensitive regions such as high latitudes and high elevations (Mountain Research Initiative EDW Working Group et al. 2015; O'Reilly et al. 2015; Sadro et al. 2019). While there is a general consensus that warming strongly accelerates methanogenesis and CH_4 emissions (Marotta et al. 2014; Yvon-Durocher et al. 2014; Sepulveda-Jauregui et al. 2018), the response of CH_4 oxidation to warming is inconsistent, as it also depends on CH_4 and oxygen (O_2) availability (Harrits and Hanson 1980; Martinez-Cruz et al. 2015; Thottathil et al. 2019). The effect of temperature on paradoxical CH_4 production in the water column is entirely unknown, as this is a currently developing research area with many uncertainties. Addressing how warming alters microbial processes involved in the CH_4 cycle in the water column will help constrain whether freshwater lakes act as a positive or negative climate feedback in coming decades. If water column CH_4 production is significant and more responsive to warming than CH_4 oxidation, it would suggest that warmer temperatures will exacerbate overall lake CH_4 emissions, generating a positive climate feedback. Conversely, if CH_4 oxidation rates are more strongly affected by warming than aerobic CH_4 production, it could suggest that CH_4 emissions from freshwater lakes will decrease.

Currently, CH_4 production under oxic conditions has been linked to several possible pathways and mechanisms, none of which are well understood. Phytoplankton are hypothesized to produce several methylated compounds that potential CH_4 producers use; moreover, they may provide anoxic environments where methanogenesis can take place (Oremland 1979; Traganza et al. 1979; Angelis & Lee 1994; Karl & Tilbrook 1994; Grossart et al., 2011). A recent study also identified members of the Cyanobacteria phylum as widespread CH_4 producers (Bižić et al., 2020). The mechanism by which Cyanobacteria produce CH_4 is currently unknown, but it is hypothesized to be related to the photosynthesis pathway. Another proposed hypothesis is that microorganisms release CH_4 by cleaving the C-P bond of methyl phosphonate (MPn) molecules for phosphorus acquisition (Karl et al. 2008; Yao et al. 2016; Wang et al. 2017; Li et al. 2020). Our previous findings showed that members of the *Comamonadaceae* family are responsible for MPn degradation in the Sierra Lakes where this study took place, and we also identified Cyanobacteria as significant contributors to aerobic CH_4 production in a mechanism involving chlorophyll metabolism (Chapter 3).

Here we investigate how increasing temperature will affect paradoxical CH_4 production and consumption in freshwater lakes. We determined whether widespread

CH_4 production or consumption occurs in the surface water of high-elevation lakes, how CH_4 production and consumption rates respond to experimentally increased temperature, whether this response was dependent on the degree of warming, and, if not, which factors contribute to CH_4 production or consumption. We designed an experimental approach to answer these questions via lake water incubations conducted at different temperatures, and combined CH_4 measurements and metagenome and metatranscriptome sequencing to assess how CH_4 producing mechanisms respond to increased temperature. We measured CH_4 concentrations over time and analyzed the abundance of functional genes of the microorganisms involved in methane paradox. Our overarching hypothesis was that most paradoxical methane production mechanisms will not be highly-temperature sensitive, as they are primarily dependent on the availability of other resources, resulting in a mosaic of warming responses occurring across experiments.

4.3 Materials and methods

4.3.1 Field site, sample collection, and experimental set up

Water samples were collected in 2016-2018 in three high-elevation lakes in Yosemite National Park, and used in incubation experiments. Samples were collected in Lukens (L), Upper Cathedral (UC), and Lower Gaylor (LG) Lakes; incubations are denoted by lake abbreviation and sequential numbering (Table 4.1). Water samples were collected in the littoral and limnetic zones of the lakes at 0.1 m depth with previously acid-washed plastic cubitainers, and then kept on ice or refrigeration to maintain in-lake temperature until laboratory incubations were established within 24 hours of samples collection. Temperature and dissolved oxygen were measured at the time of sample collection using a ProODO YSI probe (YSI Inc., Yellow Springs, OH, USA).

Water collected in the lakes was transferred to 170 or 300 ml Wheaton bottles, capped and crimped, and a known volume of air was introduced to generate a headspace for sampling. Initial CH_4 samples were collected, and bottles were incubated in water baths at constant temperature. All incubations were run in triplicate and set to the temperature at which water samples were collected in the field. The different treatments used in the experiments were:

- Control: unamended lake water following a natural day-night set up (water bath lid was opened at 7:00 hours and closed at 18:00 hours).
- Warming: unamended lake water following the same natural day-night set up than the control and in addition temperature was increased by 2°C, 4°C, 6°C or 10°C above sample collection depending on the experiment. These temperatures were chosen to simulate the response to different IPCC projections for 2099 (2°C, 4°C, 6°C; IPCC, 2014) and one extreme warming scenario (10°C).

For all incubation types, gas samples were taken from the headspace every 6-24 hours for up to 96 hours with a syringe and immediately transferred to exetainers for later analyses in a gas chromatograph. Not all treatments were tested in each incubation experiment. Temperature and oxygen concentrations were monitored at each sampling point. Optical sensor spots (Fibox, Loligo Systems, Viborg, Denmark) were used to measure oxygen concentrations during incubations (detection limit of 100 nM) and make sure that the water did not go anoxic at any time. Temperature was measured with a Fibox temperature sensor and kept constant during the incubation time. Water samples were filtered at the beginning and end of the incubation for DNA and RNA sampling.

4.3.2 Methane measurements

Methane concentrations were measured via headspace equilibration and gas chromatography. Headspace gas samples from incubations were collected with a gas-tight syringe into 12-mL Labco Exetainer vials (Labco Ltd., Lampeter, Ceredigion, UK) after incubations bottles were shaken for 2 minutes to reach equilibration. Samples were later analyzed using a Shimadzu GC-2014 gas chromatograph with flame ionization detection (FID) for CH_4. Headspace CH_4 concentration measurements were then used to calculate CH_4 concentration in lake water based on Henry's law of equilibrium (Yamamoto et al. 1976).

4.3.3 DNA and RNA extraction

Water samples were filtered through 0.22 μm (Millipore, Darmstadt, Germany) then DNA filter samples were preserved in Sucrose-Tris-EDTA (STE) buffer in pre-prepped Lysis Matrix E tubes and frozen at -80°C until extraction. RNA samples were preserved in RNA*later*® (Ambion™, AM7021) in pre-prepped Lysing Matrix E tubes (MP Bio, Eschwege, Germany), and frozen at -80°C until extraction. DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit with a modified protocol from Beman et al. (2012). Briefly, samples were lysed with 100μL 10% sodium dodecyl sulfate (SDS), DNA gets separated from proteins and cellular debris using proteinase K (20mg mL^{-1}; Qiagen, Inc., Valencia, CA, USA), precipitated with ethanol and cleaned up. After extraction samples were preserved at -80°C until further analyses. RNA was extracted using a *mir*Vana miRNA Isolation Kit (Ambion™, AM1560) with a modified protocol from Huber & Fortunato (2017). Briefly, samples are lysed with the kit's lysing matrix, then subjected to an organic extraction with phenol chloroform followed by a wash to obtain RNA. Immediately after RNA extraction we used the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies Corporation, Carlsbad, CA, USA) to synthesize first-strand cDNA and samples were preserved at -80°C until further analyses. DNA, RNA and cDNA purity was measured using a Biospectrometer (Eppendorf AG, Hamburg, Germany) and the concentrations were quantified using PicoGreen Quant-iT dsDNA quantitation assay (ThermoFisher Scientific, USA) for DNA samples and the MaestroNano Pro (Maestrogen Inc., Taiwan) for RNA and cDNA samples.

4.3.4 Metagenomes and metatranscriptomes

Metatranscriptomes and metagenomes were generated from later experiments in order to examine potential production mechanisms and coupled methane oxidation. Following extraction, DNA and RNA samples were sent for metagenome/metatranscriptome sequencing in the Vincent J. Coates Genome Sequencing Laboratory (GSL) at the University of California, Berkeley (https://genomics.qb3.berkeley.edu/), which is supported by NIH S10 OD018174 Instrumentation Grant. For each DNA sample, 250 ng of genomic DNA was sheared and libraries were prepared using the KAPA HyperPrep Kit (Kapa Biosystems, Wilmington, MA, USA). For each RNA sample, ~800 ng of total RNA was depleted of rRNA using the Ribo-Zero rRNA Removal Kit (Illumina, Inc., San Diego, CA, USA), sheared, and libraries were prepared using the KAPA RNA HyperPrep Kit (Kapa Biosystems, Wilmington, MA, USA). 7 samples were pooled into a single lane and sequenced via 150-cycle paired-end sequencing on the Illumina HiSeq 4000 platform (Illumina, Inc., San Diego, CA, USA).

Data were demultiplexed by the GSL and reads were filtered and trimmed using BBDuk (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/) with the following parameters: maq=8, maxns=1, minlen=40,minlenfraction=0.6, k=23, hdist=1, trimq=12, qtrim=rl. Forward and reverse reads were then merged using PANDASeq (https://github.com/neufeld/pandaseq; Masella et al. 2012) with default parameters. Merged reads were subsequently annotated in DIAMOND (http://diamondsearch.org/; Buchfink et al. 2015) using the NCBI NR database (accessed February 11th, 2020) with the following search criteria: maximum number of target sequences = 1, bit-score > 40. In order to quantify functional gene abundances, we filtered the DIAMOND annotation data (Percent identity > 60%, bit score > 100) to find the number of functional genes of interest present in each metagenome or metatrascriptome. We calculated percent abundance based on the total number of reads and the number of targeted genes in each metagenome or metatranscriptome.

Assembled reads were also annotated using MG-RAST (Meyer et al. 2008). MG-RAST annotated metagenomes and metatranscriptomes were run through the analysis_counter.py script of the SAMSA pipeline (Westreich et al. 2016), the output provides % abundance of different taxonomic groups and this was used for quantification and comparison in the different experiments.

4.3.5 Statistical analyses

Spearman's correlation test was used to assess the relationship between of time (h) and CH_4 concentration in all incubations and across treatments. A priori significance level was defined as $\alpha<0.05$. A significantly positive relationship between time (24 h) and CH_4 was considered as net CH_4 production in the incubation, whereas a significantly negative relationship was considered as net CH_4 consumption, otherwise

we defined the incubation as not having net CH_4 production or consumption over time. We used analysis of variance (ANOVA) and Tukey honestly significant difference (HSD) post-hoc tests to test for cases where CH_4 production or oxidation vary between sampling time points, resulting in nonlinear patterns in CH_4 concentrations over the course of the experiment for both the control and the warming treatment. For example, initial CH_4 production could be followed by subsequent oxidation, resulting in significant increases followed by significant decreases. Production or consumption rates were calculated by dividing mean net CH_4 produced or consumed by 24 hours.

To express the different responses in terms of CH_4 concentration over time among the different experiments treatments we first calculated the response ratios (lnRR) by the following equation:

$$lnRR = \ln \frac{mean\ CH_4\ in\ treatment}{mean\ CH_4\ in\ control}$$

We further used ANOVA to test for significant differences in CH_4 concentration between the control and the warming treatment at 24 hours. Moreover, we used two-way ANOVA to test the combined effect of treatment and time point on CH_4 concentrations. All statistical analyses and figures were done in the R statistical environment (RStudioVersion 1.2.5001).

4.4 Results and discussion

4.4.1 Effect of warming on aerobic methane production and consumption

Methane production and oxidation were both active and dynamic across our experiments. Out of twelve experimental incubations, we observed both net CH_4 production and net consumption after 24 hours in both control and warming treatments. Within the control incubations, 33% of experiments showed significant CH_4 production (L1, L2, LG3, UC1), while 16% showed significant CH_4 consumption (L5, L7) (averaged across replicate experimental bottles incubated for 24 hours; Fig. 4.1). The remaining experiments displayed no net significant change over the experiment (50%; LG1, LG2, LG5, LG6, UC3, UC4). However, three of these experiments displayed statistically significant differences in CH_4 concentrations among different time points in experiments (LG1, LG6, and UC3). The lack of a linear trend in these experiments, but the presence of significant variation over time, indicate differences in the balance between CH_4 production and consumption over time.

Experiments under elevated temperatures exhibited great variation in CH_4 within replicates and among experiments; statistically significant production occurred in 33% of the experiments (LG1, LG3, UC1, UC4), with significant consumption in one of the experiments (LG5; Fig. 4.1). Of the remaining experiments showing no significant net change after 24 hours, L7 and LG6 displayed statistically significant variation based on ANOVA—suggesting changes in microbial CH_4 production and

oxidation over time in these experiments. Although CH_4 production was variable across experiments, overall there were more incubations with net CH_4 production, and fewer showing CH_4 consumption in the warming treatment. Collectively, results from the control and warming treatments confirm paradoxical CH_4 production in freshwater lakes.

We used experimental warming to examine the effects of temperature change on overall CH_4 production and consumption, and observed a warming effect in nine experiments. Experiments L5, L7, LG1 and UC4 showed considerably higher CH_4 concentrations in the warming treatment. Moreover, in the L5 and LG1 experiments, we observed a switch from net CH_4 consumption to potential CH_4 production. Methane concentrations in experiment L7 increased 93% under the warming treatment and over 2000% in UC4. These overall changes may reflect increased CH_4 production, decreased CH_4 oxidation, or a combination of the two—and we examined the underlying dynamics using metagenomics and metatranscriptomics. In contrast, the L1, L2, LG2, LG3, and LG5 experiments showed notably lower CH_4 concentrations—indicating decreased production, increased oxidation, or both. Production rates decreased 29% with warming in L1, 53% in L2, and over 90% in LG3. In both LG2 and LG5, the warming treatment showed significant CH_4 consumption compared to potential production in the control ($p<0.05$; Table 4.1). As these data indicate, we did not observe consistent patterns in the response of overall CH_4 production and consumption to different levels of warming. For example, in the experiments where we saw significant CH_4 increase in warming treatments over 24 hours, one was in response to a 10 °C increase (LG1) two to a 4°C increase (L7 and UC4), and one to a 2 °C increase (L5). In contrast, in the experiments with CH_4 decreases, L1 and LG2 showed significant decreases at 10 °C, L2 at 6°C, LG3 at 4°C and LG5 at 2°C (Table 4.1, Fig. 4.2).

4.4.2 Disentangling the effects of warming on aerobic methane production and consumption mechanisms

To further disentangle whether observed changes in overall CH_4 concentrations are due to increases or decreases in CH_4 production or consumption, we generated metagenomes and metatranscriptomes from three experiments, and measured the response to warming of several functional genes involved in CH_4 cycling. We examined three experiments that showed a full range of responses: the LG2 experiment displayed significant consumption in the warming treatment, the LG6 experiment showed no significant differences between the warming treatment and the control, and the UC4 experiment exhibited CH_4 increase in the warming treatment but not in the control. We identified functional genes related to potential processes in the aerobic CH_4 cycle and surveyed them in metagenomes and metatranscriptomes. These included the phosphonate-binding periplasmic protein (*phnD)* and alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase *(phnJ)* genes (for their role in phosphonate assimilation and breakdown of MPn respectively; Rizk et al. 2006; Repeta et al. 2016) and methane monooxygenase (*mmo*) and particulate methane monooxygenase (*pmoA* genes (for their role in methane oxidation; Samad and

Bertilsson 2017). We also examined changes in ferredoxin:protochlorophyllide reductase (DPOR) and chlorophyllide a reductase (COR) for their potential role in Cyanobacteria and photosynthetic bacteria CH_4 production during the metabolism of chlorophyll as found in our previous study (Chapter 3). Finally, we quantified all reads from two main bacterial groups implicated in CH_4 production, the *Comamonadaceae* and the Cyanobacteria.

In the LG2 experiment, we observed significant CH_4 consumption in response to 10°C warming. Metagenomes were sequenced from the beginning of the incubation (0 h) and at the end (24 h) of experiment from both the control (LG2 – tf C) and the warming treatment (LG2 -tf W). In this experiment, we saw a considerable increase in both the *mmo* and *phnD* genes, and only small decreases in all the other functional genes measured (Fig. 4.3). In terms of taxon abundances, members of the *Comamonadaceae* family decreased slightly in the control but remained in similar abundances in the warming treatment (Fig. 4.4). For the Cyanobacteria phylum, the LG2 metagenome showed a decrease over time in both the control and the warming treatment (Fig. 4.4). Metagenomes are likely to show less intense responses to experimental treatments compared to metatranscriptomes, but still offer insight into microorganisms and functional gene differences in response to treatment. The slight decrease in gene abundance related to paradoxical CH_4 production mechanisms (*phnJ*, DPOR and COR) and producers suggests that warming did not strongly influence aerobic CH_4 production here—however, it may have positively impacted methane oxidation as shown by the increase of *mmo* genes. This is further consistent with decreased CH_4 concentrations observed under warming.

The LG6 experiment was subjected to a 4 °C increase and showed no significant differences in CH_4 concentrations over time in the warming treatment compared to the control. Underlying this, transcripts related to aerobic CH_4 production and consumption mechanisms had complex responses in this experiment. RNA sampling occurred at the beginning (0 h) and end (96 h) of the warming experiment, and *phnD* transcript abundance decreased while *phnJ* abundance increased significantly (Fig. 4.3). DPOR and COR both showed small increases in abundance. In terms of methane oxidation, we observed a substantial decrease in the expression of *pmoA* in the warming treatment and a significant increase (over an order of magnitude) in the *mmo* expression. The abundance of *Comamonadaceae* transcripts decreased almost 50% in this experiment. Similarly, we observed reduced cyanobacterial transcripts by the end of the warming treatment. These mixed responses may reflect a balance between the mechanisms involved in aerobic CH_4 production and consumption in response to warming, leading to a lack of change among the control and the warming treatment.

Finally, in the UC4 experiment, a 4 °C increase generated a distinct increase in CH_4 concentration compared to the control. We sampled RNA at the beginning (0 h) and end (96 h) of the experiment. In contrast with the previous experiments, all the transcripts measured here increased by the end of the warming treatment. However, DPOR and COR had both over an order of magnitude of increase compared with more modest increases in *pmoA*, *mmo*, *phnD* and *phnJ*. Both comamonadaceael and cyanobacterial transcripts decreased by the end of the warming experiment. Our

results suggest that in this experiment, aerobic CH_4 production responded positively to warming—especially in the pathway being carried out potentially by Cyanobacteria regardless of the final cyanobaterial transcript abundance. Furthermore, the observed increases in *pmoA* and *mmo* transcripts suggest a similarly positive response in CH_4 oxidation.

4.4.3 General patterns in functional genes and taxonomic groups in response to warming

We observed both aerobic CH_4 production and consumption in our experiments, and both were affected by experimental warming. The varied effects of warming on CH_4 can be explained by the individual responses of the mechanisms involved in aerobic CH_4 production and CH_4 oxidation, and their relative balance. For example, we would expect net CH_4 production rates if microbial production surpasses CH_4 consumption; this could be the result of increased CH_4 production under higher temperatures combined with a negative effect—or a weaker positive effect—of warming on CH_4 oxidation. Conversely, if positive response to warming for CH_4 oxidation is higher compared to a weaker positive effect or a negative effect on CH_4 production, we would observe net CH_4 consumption.

Across experiments, *mmo* genes and transcripts involved in CH_4 oxidation showed a positive response to warming by increasing in abundance. While there is evidence of a positive response in CH_4 oxidation to warming (Bastviken 2009; Duc et al. 2010; Shelley et al. 2017; Sepulveda-Jauregui et al. 2018; Thottathil et al. 2019) there are complex interactions at play. Methane oxidation response to warming has been documented to be subject of CH_4 and O_2 concentrations (Liikanen et al. 2002; Guérin & Abril, 2007; Lofton et al. 2014; Martinez-Cruz et al. 2015; Thottathil et al. 2018). For example, the effect of warming on CH_4 oxidation may be stronger when CH_4 concentrations are higher but may be unaffected by higher temperatures when CH_4 concentrations are lower (Lofton et al. 2014; Shelley et al. 2017; Fuchs et al. 2016). Low O_2 concentrations can also inhibit CH_4 oxidation (Rudd et al. 1976), but O_2 concentrations were elevated through all our incubations (240 to 390 umol/l), so CH_4 oxidation activities in our experiments are more likely to be limited by CH_4 concentration. LG2 concentrations were below 40 nM at all times, LG6 concentrations ranged between 100 and 120 nM, and UC4 concentration ranged between 40 and 60 nM. In those incubations with the lowest CH_4 concentrations (LG2 and UC4 control; all <40 nM) the metagenome and metatranscriptome data did not show presence of *pmoA* genes or transcripts, suggesting that the activity may have been limited by CH_4 concentration. Comparably, Fuchs et al. (2016) study showed that the relative abundance *pmoA* genes from lake sediment and water incubations had no statistical differences under different temperature incubations. They found that *pmoA* abundances were instead correlated to CH_4 concentrations. While we do not have metagenomes or metatranscriptomes from all experiments, changes in CH_4 over time can help discern if higher CH_4 concentrations promote higher consumption rates. Experiments with higher initial CH_4 concentrations (<100 nM; L1, L2, L5, L7, LG5 and LG6) showed inconsistent patterns. In L1, L2 and LG5 warming treatment

resulted in lower CH_4 concentrations compared to the control suggesting increased consumption. L5, L7 and LG6, showed the opposite effect where CH_4 concentrations were higher in the warming treatment. Furthermore, our data show that CH_4 oxidation may still be taking place as observed in experiments with lower concentrations (CH_4 < 100 nM; LG2, LG3, UC3). Overall, we observed potential CH_4 consumption in our experiments at a wide range of CH_4 concentrations (10 - 600 nM) when subjected to increased temperatures. Moreover, increases in the abundance of *mmo* genes and transcripts in our experiments L2, LG6 and UC4 suggest some temperature sensitivity of methane oxidation. Ultimately, our results suggest a positive CH_4 oxidation response to warming that can still occur under low CH_4 concentrations.

Aerobic CH_4 production via MPn metabolism was active in these experiments and showed a consistently positive response to warming. *PhnJ* expression was higher in both metatranscriptomes, and it only decreased in gene abundance in the LG2 experiment (Fig. 4.3). Similarly, *phnD* gene and transcript was higher in the LG2 and UC4 experiment but lower in LG6 (Fig. 4.3). Moreover, abundances of *Comamonadaceae* family members in our experiments slightly decreased in the warming treatment (with the exception of the LG6 experiment where we saw significant decrease in the transcript abundances). As a newly discovered methane production mechanism, we are not aware of any studies published on the warming response of *phnJ* gene expression or abundance. A study by Ma et al. (2020) showed that abundances of *Limnohabitans* and *Polynucleobacter* OTUs, two important genera in the microbial communities of the lakes here studied (Chapter 3), decreased during an abnormally cool period in an alpine lake. The same study also found a positive correlation between air temperature and the abundance of *Limnohabitans* and an unknown genus of *Comamonadaceae*. These findings suggest that warming has a positive effect on the abundance on some genera within the *Comamonadaceae* family.

Ultimately, *phnJ* and *phnD* expression may be primarily regulated by phosphorus limitation—such that temperature may be secondary to the effect of nutrient changes in lakes (nutrient depletion in our experiments may also be the reason for the consistent decrease in *Comamonadaceae* gene abundance, as lake water collected was already oligotrophic at the beginning of the experiment). Nonetheless, we show that *phnJ* and *phnD* were upregulated in experimental warming treatments, and that this was coincident with significant CH_4 production (UC4 experiment). Our findings suggest that CH_4 production due to phosphonate assimilation may be temperature sensitive, perhaps reflecting higher nutrient demands to sustain higher metabolic rates under elevated temperature.

Cyanobacteria have been proposed to be able to produce CH_4 under aerobic conditions and given they are ubiquitous this can have global implications (Bižić et al. 2020). The overall consensus is that increases in temperature will positively impact cyanobacterial growth, especially in those species causing detrimental cyanobacterial blooms (Elliott 2010; Kosten et al. 2012; Visser et al. 2016) as their optimal growth temperature ranges from 25-30 °C (Visser et al. 2016; Verbeek et al.

2018). Nonetheless, their growth may be dependent on nutrient concentrations as well as temperature increases (Elliott 2010; Verbeek et al. 2018). In our study we observed Cyanobacteria decreasing over time and in the warming treatment. Overall, a negative response to warming (in terms of abundance and gene expression) like the observed in these experiments could be related to the low nutrient concentrations in our incubations. Bižić et al. (2020) did not identify the mechanism by which Cyanobacteria produce CH_4, but our results showed that DPOR and COR could be involved in the mechanism of CH_4 production in Cyanobacteria, photosynthetic bacteria and green algae (Chapter 3). DPOR and COR expression was increased by the warming treatment particularly in the UC4 experiment where we saw increased production only under the warming treatment. This supports CH_4 production during porphyrin and chlorophyll metabolism, but most importantly, our results show that this CH_4 production mechanism could significantly increase CH_4 emissions as global temperatures increase.

In this study we showed that aerobic CH_4 production occurs in high-elevation freshwater lakes and can be altered by experimental warming. We observed a significant warming effect (positive or negative) in 75% of the experiments, and we were able to attribute CH_4 changes over time and within warming treatment to the gene expression and abundance of functional genes in metatranscriptomes and metagenomes from three of the experiments (LG2, LG6 and UC4). In particular, we observed increased abundances of functional genes and transcripts involved in paradoxical CH_4 production (*pnnJ,* DPOR and COR*)* under warming. *mmo* genes and transcripts increased with warming in all the metagenome and metatranscriptome data, but *pmoA* response differed. Our results therefore demonstrate that both CH_4 production and CH_4 oxidation genes can be upregulated under elevated temperatures, indicating that warming can affect both methane production and consumption. Imbalances between these two processes in their warming responses would lead to conflicting overall patterns in CH_4. Overall, 33% of experiments were positively affected, while 41% showed an overall negative response. Ultimately, our results show that aerobic CH_4 production responds positively to warming, and suggest that as global temperature increases, so will CH_4 emissions derived from paradoxical CH_4 production.

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4.6 Tables

Table 4.1 Experimental incubations conditions. Asterisks denote incubations that were significantly different in CH_4 concentrations under the warming treatment at 24 hours.

Experiment	Y	M	D	Initial T (°C)	Increase in T(°C)
L1	2016	8	12-13	20	10
L2	2016	9	11-12	18	6
L5	2017	9	26-29	14	2
L7	2018	9	23-28	14	4
LG1	2016	8	8-9	18	10
LG2*	2016	9	9-10	15	10
LG3	2016	10	7-8	8	4
LG5*	2017	10	1-4	10	2
LG6	2018	9	10-14	16	4
UC1	2016	8	10-11	18	10
UC3	2017	10	8-11	10	2
UC4	2018	10	8-12	10	4

4.7 Figures

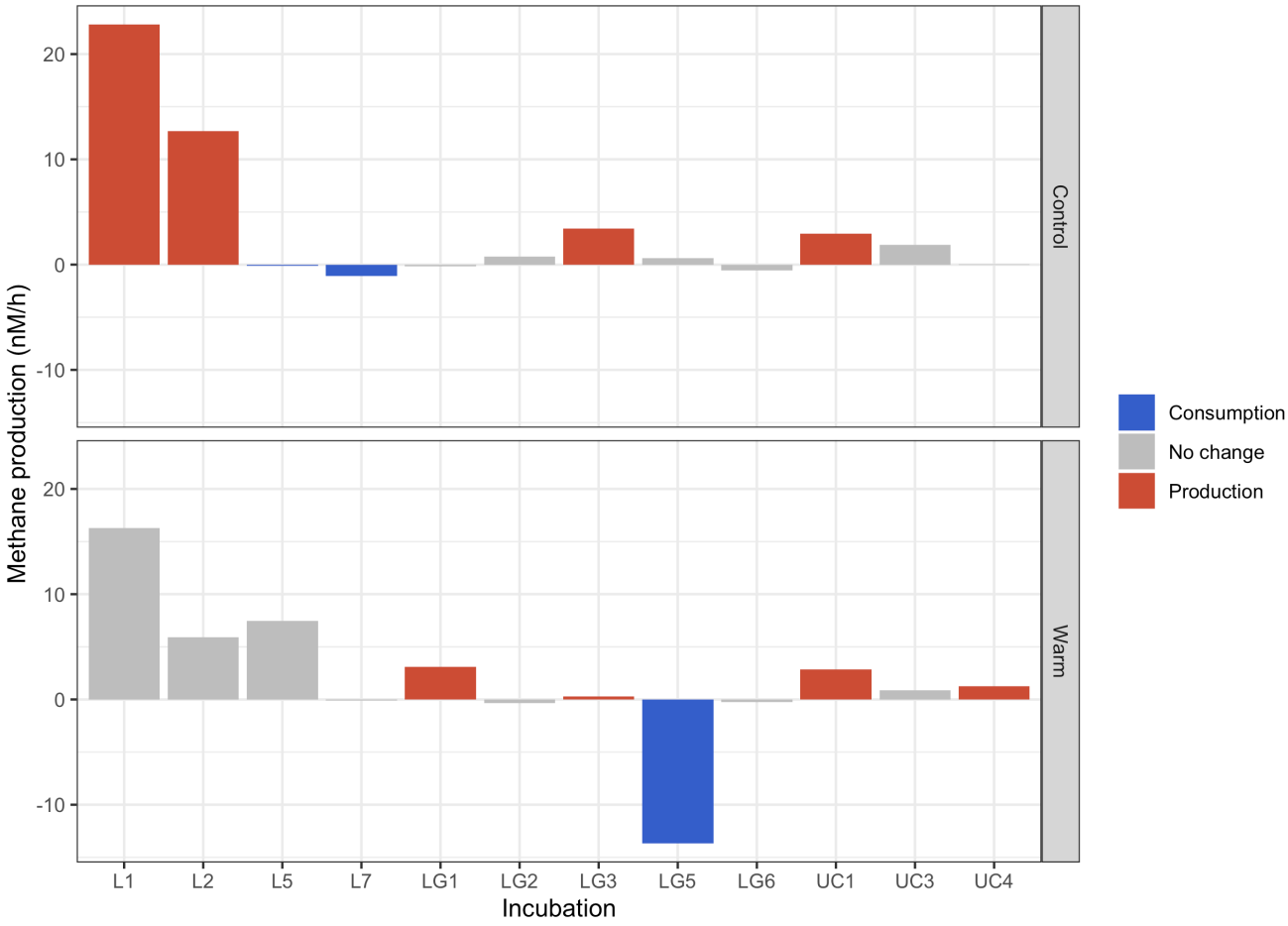

Figure 4.1 Methane production and consumption rates by incubation experiment. Different colors represent production, consumption or no significant change in CH_4 concentrations over 24 hours. Top panel represents the rate of production or consumption in control incubations. Bottom panel represents the rate of production or consumption under warming treatment.

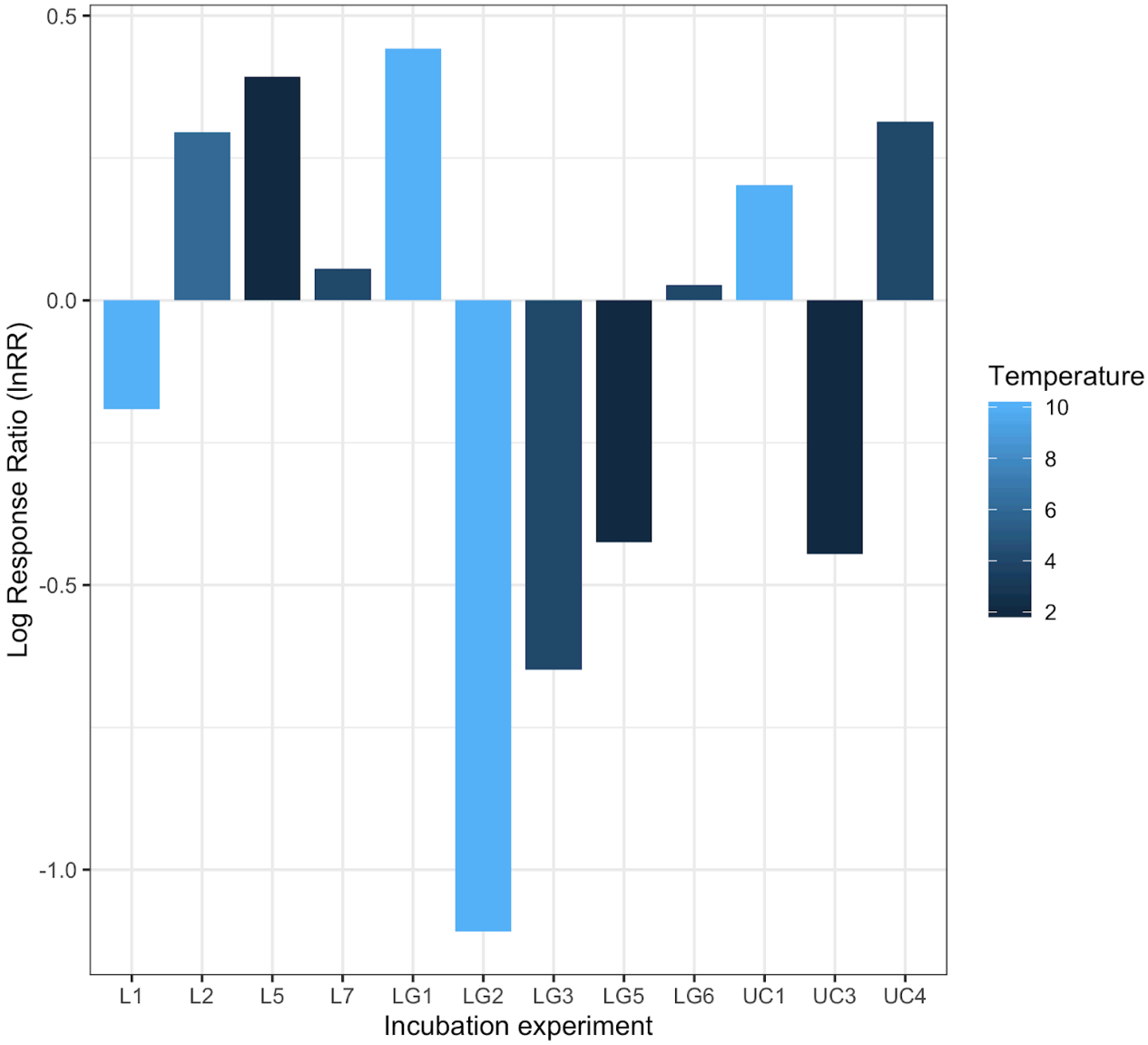

Figure 4.2 Treatment effect of mean methane produced or consumed on the experiments. Different shades of blue represent different experimental temperature increase. *High variability in CH_4 concentrations within replicates results in differences in the effect of warming between lnRR and consumption rates in L2 experiment.

Figure 4.3 Gene and transcript change (%) in total reads of metagenomes and metatranscriptomes in warming treatments compared to controls. Change in (a) gene abundance in LG2 experiment, (b) transcript abundance in LG6 experiment, and (c) transcript abundance in UC4 experiment for functional genes involved in phosphonate assimilation (*phnD*, *phnJ*), methane oxidation (*mmo*, *pmoA*) and porphyrin and chlorophyll metabolism (DPOR: ferredoxin:protochlorophyllide reductase and chlorophyllide a reductase: COR).

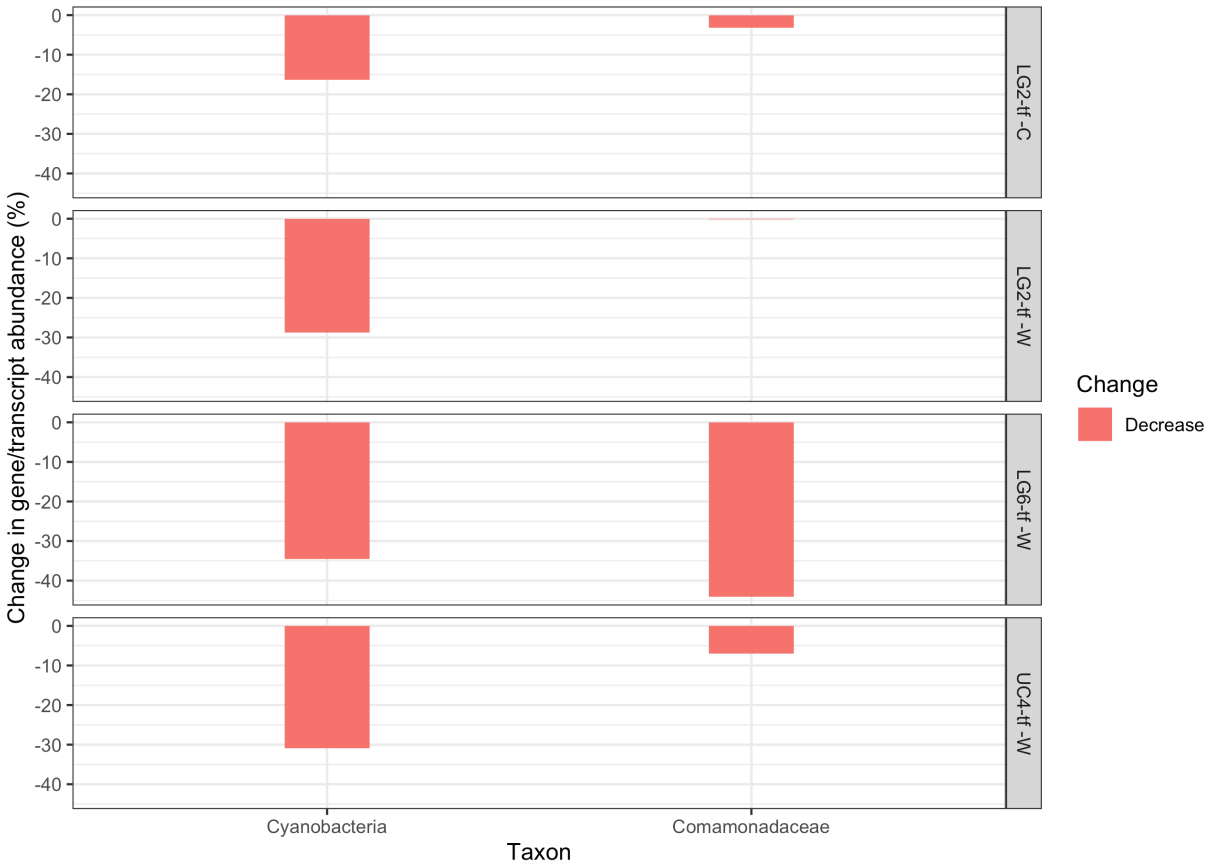

Figure 4.4 Gene and transcript change (%) in total reads of metagenomes and metatranscriptomes in warming treatments compared to controls. Change in (a) gene abundance in LG2 experiment, (b) transcript abundance in LG6 experiment, and (c) transcript abundance in UC4 experiment for members of the *Comamonadaceae* family and the Cyanobacteria phylum

5 Conclusions

The objective of this research project was to understand the dynamics involved in the methane (CH_4) cycling of high-elevation freshwater lakes. Specifically, this work's purpose was to measure seasonal changes in CH_4 concentration and fluxes and find relevant environmental parameters that could best explain these seasonal variations. Another important aim of this work was to elucidate the mechanisms and players involved in the methane paradox of freshwater lakes via several experimental treatments and combined biogeochemical measurements, 16S rRNA, metagenome, and metatranscriptome sequencing and stable isotopes analysis. Finally, another major objective of this work was to investigate the effect of temperature increases on high-elevation CH_4 cycling and specifically in those biological mechanisms involved in the methane paradox. The main findings of this research are summarized below:

Chapter 2 results showed high seasonal variability in CH_4 concentrations and fluxes which highlights the importance of accounting for temporality in predictions of future CH_4 emissions. Moreover, analysis of environmental parameters measured over two years revealed that elevation could best explain CH_4 concentrations among all the lakes sampled, nonetheless, different environmental parameters influenced CH_4 concentrations at different elevations. In the three lakes at elevations below 3000m, temperature and nitrite were the best predictors of CH_4 concentration whereas at higher elevations (>3000m) CH_4 was better predicted by changes in DIN:DIP ratios. Overall, while temperature had a positive effect on CH_4 concentrations this was only significant at lower elevations, whereas at higher elevation changes in nutrient sources appeared to have a more significant effect.

Chapter 3 findings showed that paradoxical methane production was significant in our experiments. Moreover, through the use of different experimental treatments, 16S rRNA, metagenome, and metatranscriptome sequencing, and stable isotope analysis it was possible to identify the main mechanisms active in the water column of these lakes. Predominantly, CH_4 production could be attributable to methylphosphonate (Mpn) breakdown whereas methanogenesis was an unlikely mechanism for CH_4 production in the experiments. Moreover, cyanobacterial and protebacterial CH_4 production was likely active in some of the experiments and the mechanism by which they produced CH_4 is proposed to happen during the metabolism of porphyrin and chlorophyll. This represents a new potential aerobic CH_4 production mechanism that has not been previously described.

Chapter 4 showed that even though the effect of the warming treatment on the abundances of functional genes and transcripts involved in aerobic CH_4 mechanisms was variable, these changes in abundance could be used to explain changes in CH_4 concentrations over time. Moreover, Mpn breakdown and potential cyanobacterial CH_4 production were positively responsive to increased temperature which suggests increased emissions from lakes in a warmer future.

Overall, this Ph.D. research project showed that CH_4 cycling is highly variable, both temporally (over seasons) and spatially (elevation gradient) and it is heavily influenced by changes in temperature, nutrients and dissolved organic carbon. As we move forward with research in this field, it has to become a priority to better understand all the sources producing CH_4 as well as how they are and will be affected by changes in the environment. This work contributes the quantification of seasonal variations in CH_4 in understudied high-elevation lakes. Moreover, this study elucidates the paradoxical CH_4 production mechanisms active in these lakes, identifying one new mechanism with potential of being active and widespread in aquatic ecosystems by Cyanobacteria and Proteobacteria. Alongside, this research measured the potential effects of increased temperatures both in the field and in laboratory incubations finding that the although responses were variable, warming seemed to impact both CH_4 producer and consuming microorganisms. Constraining CH_4 budgets in a changing world is a challenge that can only be achieved by completely understanding all the potential sources and sinks of CH_4 and how they respond to environmental change, further advancement of the field requires interdisciplinary research that bridges field and laboratory approaches, 'omics and modelling to get a clearer picture of how CH_4 cycling will look in a warmer future.